BACTERIAL EFFECTOR PROTEINS WHICH INHIBIT PROGRAMMED CELL DEATH

[0001] The present invention claims benefit of U.S. Provisional Application Serial No. 60/404,339, filed August 16, 2002, and U.S. Provisional Application Serial No. 60/425,842, filed November 12, 2002, which are hereby incorporated by reference in their entirety.

[0002] The subject matter of this application was made with support from the United States Government under the United States Department of Agriculture NRI Grant No. 99-35301-7973 and the National Science Foundation Grant No. DBI-0077622. The Government may have certain rights.

FIELD OF THE INVENTION

[0003] The present invention relates to a bacterial effector protein which inhibits programmed cell death ("PCD") in eukaryotes.

BACKGROUND OF THE INVENTION

[0004] *Pseudomonas syringae* pv. *tomato* DC3000 is a widely studied model plant pathogen that causes disease on tomato and *Arabidopsis*. DC3000 uses a type III secretion (TTSS) system to directly deliver bacterial effector proteins into the host cell (Galan et al., "Type III secretion machines: Bacterial devices for protein delivery into host cells." *Science*, 284: 1322-1328 (1999)). Loss of function mutations in the TTSS completely abrogate *P. syringae* disease formation, indicating that effectors are essential agents of *P. syringae* pathogenesis (Collmer et al., "Pseudomonas syringae Hrp type III secretion system and effector proteins." *Proc Natl Acad Sci USA*, 97: 8770-8777 (2000)). In bacterial pathogens of plants, the TTSS is encoded by the hypersensitive response ("HR") and pathogenicity (*hrp*) genes (Lindgren, P. B., "The role of *hrp* genes during plant-bacterial interactions." *Annu. Rev. Phytopathol.* 35: 129-152 (1997)). Mutations in key *hrp* genes prevent the secretion of effectors and inhibit pathogen growth and host defenses. A hallmark of effector genes is the presence

of a "Hrp box" cis element in their promoter which is recognized by the HrpL ECF-like sigma factor (Innes et al., "Molecular analysis of avirulence gene *avrRpt2* and identification of a putative regulatory sequence common to all known *Pseudomonas syringae* avirulence genes." *J. Bacteriol.* 175: 4859-4869 (1993); Xiao et al., "Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonase syringae* pv. *syringae* Pss61 *hrp* and *hrmA* genes." *J. Bacteriol.* 176: 1025-1036 (1994)). A recent search for Hrp box containing genes in the genome of *Pseudomonas syringae* pv. *tomato* strain DC3000 revealed over 20 putative effector genes (Fouts, et al., "Genomewide identification of Pseudomonas syringae pv. tomato DC3000 promoters controlled by the HrpL alternative sigma factor." *Proc Natl Acad Sci U S A*, 99: 2275-2280 (2002)). Although the role of effector proteins in pathogen virulence is poorly understood, many effectors have been isolated based on their ability to trigger host immunity.

[0005] In the "gene-for-gene" model of plant immunity, disease resistance is initiated by recognition of a pathogen avirulence (Avr) effector protein by a plant resistance (R) protein. The tomato R protein Pto, a serine/threonine protein kinase, recognizes and directly interacts with DC3000 effector proteins AvrPto and AvrPtoB, and initiates immunity in tomato by characterized and uncharacterized signaling mechanisms (Kim et al., "Two distinct pseudomonas effector proteins interact with the pto kinase and activate plant immunity." *Cell*, 109: 589-598 (2002); Scofield et al., "Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato." *Science*, 274: 2063-2065 (1996); Sessa et al., "Signal recognition and transduction mediated by the tomato Pto kinase: a paradigm of innate immunity in plants." *Microbes Infect*, 2: 1591-1597 (2000); Tang et al., "Overexpression of Pto activates defense responses and confers broad resistance." *Plant Cell*, 11: 15-30 (1999)). Interestingly, the Pto kinase shares sequence similarity with the human interleukin-1 receptor associated kinase (IRAK) and with the *Drosophila* Pelle kinase, both of which, like Pto, play a role in immune responses (Cohn et al., "Innate immunity in plants." *Curr. Opin. Immunol.*, 13: 55-62 (2001); Hoffman et al., "Phylogenetic perspectives in innate immunity," *Science* 284:1313-1318 (1999)). The *Pto* gene belongs to a gene family of 6 members on tomato chromosome 5 (Martin et al., "Map-based cloning

of a protein kinase gene conferring disease resistance in tomato." *Science*, 262: 1432-1436 (1993); Michelmore et al., "Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process." *Genome Res.* 8: 1113-1130 (1998); Riely et al., "Ancient origin of pathogen recognition specificity conferred by the tomato disease resistance gene *Pto*." *Proc. Natl. Acad. Sci. USA* 98: 2059-2064 (2001)). One of these family members, *Fen*, encodes a kinase that confers sensitivity to an insecticide (fenthion), while the function of the others is unknown (Martin et al., "A Member of Tomato *Pto* Gene Family Confers Sensitivity to Fenthion Resulting in Tomato," *Plant Cell* 6:1543-1552 (1994)).

[0006] The *R* gene-mediated plant immune response is characterized by a series of physiological changes in the plant cell, including the formation of reactive oxygen species, induction of defense genes, and the HR. The HR is defined as a defense response involving rapid, localized cell death that functions to limit pathogen growth (Goodman et al., "The hypersensitive reaction in plants to pathogens." *APS Press, St. Paul, Minnesota, USA*, (1994)). The cell death associated with the HR is a genetically controlled and regulated process and an example of programmed cell death in plants (Greenberg, J.T. "Programmed cell death in plant-pathogen interactions." *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 48: 525-545 (1997); Heath, M.C. "Hypersensitive response-related death." *Plant Mol Biol*, 44: 321-334 (2000)). As such, programmed cell death is a hallmark of HR-based immunity in plants, and cell death phenotypes are often used in laboratory experiments to discover and dissect plant immune responses.

[0007] The AvrPtoB protein has a predicted molecular mass of 59 kDa, is secreted via the TTSS, and triggers the HR and immunity in Pto-expressing tomato plants. AvrPtoB has limited similarity to AvrPto; however, it shares 52% amino acid identity with the *P. s.* pv. *phaseolicola* effector VirPphA (Jackson et al., "Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native plasmid in the bean pathogen Pseudomonas syringae pathovar phaseolicola." *Proc Natl Acad Sci USA*, 96: 10875-10880 (1999)). In general, bacterial effector proteins are highly diverse with little amino acid sequence similarity among them (one exception is the AvrBs3 family; Lindgren, P. B., "The role of *hrp* genes during plant-bacterial interactions." *Annu. Rev. Phytopathol.* 35: 129-152 (1997); White et al., "Prospects for understanding

avirulence gene function." *Curr.* Opin. Plant Biol. 3: 291-298 (2000)). They have been identified from all four of the most common genera of plant bacterial pathogens (i.e., *Pseudomonas, Xanthomonas, Erwinia,* and *Ralstonia).* In a still cryptic process, these pathogens utilize the TTSS to inject effectors across the plant cell wall into the cytoplasm (Galan et al., "Type III secretion machines: Bacterial devices for protein delivery into host cells." *Science,* 284: 1322-1328 (1999); Jin et al., "Role of the Hrp pilus in type III protein secretion in *Pseudomonas syringae.*" *Science* 294: 2556-2558 (2001)). Little is known of the fate of bacterial effectors once they are in the plant cell although some members of the AvrBs3 family are localized to the nucleus, some effector proteins are targeted to the plasma membrane after being myristylated, and others are processed to smaller forms (Nimchuk et al., "Eukaryotic fatty acylation drives plasma membrane targeting and enhances function of several type III effector proteins from *Pseudomonas syringae.*" *Cell.* 101: 353-363 (2000); Shan et al., "The *Pseudomonas* AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane." *Plant Cell* 12: 2323-2337 (2000b); Van der Ackerveken et al., "Recognition of the bacterial avirulence protein AvrBs3 occurs inside the host cell." *Cell* 87: 1307-1316 (1996); Zhu et al., "The C terminus of AvrXa10 can be replaced by the transcriptional activation domain of VP16 from the herpes simplex virus." *Plant Cell.* 11: 1665-1674 (1999)).

[0008] The AvrPto protein and the Pto kinase physically interact in a yeast two-hybrid system (Scofield et al., "Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato." *Science* 274: 2063-2065 (1996); Tang et al., "The avirulence protein AvrPto physically interacts with the Pto kinase." *Science* 274: 2060-2063 (1996)). Co-expression of Pto and AvrPto as transgenes in a *pto* mutant leaf is sufficient to activate resistance. Mutations that disrupt this interaction also abolish the ability to elicit disease resistance in plant leaves (Chang et al., "Functional studies of the bacterial avirulence protein AvrPto by mutational analysis." *Mol. Plant-Microbe Interact.* 14: 451-459 (2001); Frederick et al., "Recognition specificity for the bacterial avirulence protein AvrPto is determined by Thr-204 in the activation loop of the tomato Pto kinase." *Molecular Cell.* 2: 241-245 (1998); Shan et al., "The *Pseudomonas* AvrPto protein is

differentially recognized by tomato and tobacco and is localized to the plant plasma membrane." *Plant Cell* 12: 2323-2337 (2000)). Resistance is dependent on the Prf protein which bears striking similarity to the large NB-LRR class of R proteins (Salmeron et al., "Tomato *Prf* is a member of the leucine-rich repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster." *Cell* 86: 123-133 (1996)). Pto-Fen chimeras were used to define the kinase activation loop as a key determinant of Pto interaction specificity for AvrPto (Frederick et al., "Recognition specificity for the bacterial avirulence protein AvrPto is determined by Thr-204 in the activation loop of the tomato Pto kinase." *Molecular Cell.* 2: 241-245 (1998); Scofield et al., "Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato." *Science* 274: 2063-2065 (1996); Tang et al., "The avirulence protein AvrPto physically interacts with the Pto kinase." *Science* 274: 2060-2063 (1996)). Pto kinase is phosphorylated on 8 residues and mutation of two of these residues (T38 and S198) abolishes its ability to elicit host resistance (Sessa et al., "Thr38 and Ser198 are Pto autophosphorylation sites required for the AvrPto-Pto-mediated hypersensitive response." *EMBO J.* 19: 2257-2269 (2000)). Recognition specificity of Pto for AvrPto appears to have evolved before *Lycopersicon* speciation because a Pto family member from a distantly related species, *L. hirsutum*, also recognizes AvrPto (Riely et al., "Ancient origin of pathogen recognition specificity conferred by the tomato disease resistance gene *Pto*." *Proc. Natl. Acad. Sci. USA* 98: 2059-2064 (2001)).

[0009] The AvrPto gene was originally isolated from *P. s. tomato* strain JL1065 based on its ability to confer avirulence to a virulent strain of *P. s. maculicola* (Ronald et al., "The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the Pto resistance gene." 174: 1604-1611 (1992)). AvrPto encodes an 18 kD protein that bears little sequence similarity to proteins in current databases (Salmeron et al., "Molecular characterization and *hrp* dependence of the avirulence gene *avrPto* from *Pseudomonas syringae* pv. *tomato*." *Mol. Gen. Genet.* 239: 6-16 (1993)). Its mechanism of activating resistance is unknown although it likely interacts with Pto inside the plant cell and possibly with certain 'AvrPto-dependent Pto-interacting' (Adi) proteins as part of a complex (Bogdanove et al., "AvrPto-dependent Pto-interacting proteins and

AvrPto-interacting proteins in tomato." *Proc. Natl. Acad. Sci. USA* 97: 8836-8840 (2000); Scofield et al., "Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato." *Science* 274: 2063-2065 (1996); Tang et al., "The avirulence protein AvrPto physically interacts with the Pto kinase." *Science* 274: 2060-2063 (1996)). AvrPto acts as a virulence factor when Pto (or Prf) is absent from the plant cell and increases the growth of *P. s. tomato* about 10-fold as compared to a strain lacking the effector (Chang et al., "*avrPto* enhances growth and necrosis caused by *Pseudomonas syringae* pv. *tomato* in tomato lines lacking either *Pto* and *Prf*." *Mol. Plant-Microbe Interact.* 13: 568-571 (2000); Shan et al., "A cluster of mutations disrupt the avirulence but not the virulence function of AvrPto." *Mol. Plant-Microbe Interact.* 13: 592-598 (2000)). In common with several effectors, AvrPto has a myristylation motif at its N terminus that is required for both its avirulence and virulence activity (Nimchuk et al., "Eukaryotic fatty acylation drives plasma membrane targeting and enhances function of several type III effector proteins from *Pseudomonas syringae*." *Cell.* 101: 353-363 (2000); Shan et al., "The *Pseudomonas* AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane." *Plant Cell* 12: 2323-2337 (2000)). The amino acids of AvrPto that are required for its recognition by the Pto kinase have been examined by saturation mutagenesis (Chang et al., "Functional studies of the bacterial avirulence protein AvrPto by mutational analysis." *Mol. Plant-Microbe Interact.* 14: 451-459 (2001); Shan et al., "A cluster of mutations disrupt the avirulence but not the virulence function of AvrPto." *Mol. Plant-Microbe Interact.* 13: 592-598 (2000); Shan et al., "The *Pseudomonas* AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane." *Plant Cell* 12: 2323-2337 (2000)). Mutation of three AvrPto residues -- S94, I96, and G99 -- abolishes interaction with Pto and avirulence activity, but not virulence activity, in tomato (Shan et al., "A cluster of mutations disrupt the avirulence but not the virulence function of AvrPto." *Mol. Plant-Microbe Interact.* 13: 592-598 (2000); Shan et al., "The *Pseudomonas* AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane." *Plant Cell* 12: 2323-2337 (2000)). Along with the other observations (Chang et al., "Functional studies of the bacterial avirulence protein AvrPto by mutational

analysis." *Mol. Plant-Microbe Interact.* 14: 451-459 (2001)), these results indicate that an internal region of AvrPto determines its binding specificity for Pto.

[0010] *AvrPto*-like DNA sequences are present in *Pseudomonas* strains that are known to be avirulent on *Pto* tomato plants (race 0 strains) and are absent from virulent ones (race 1 strains; Ronald et al., "The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the Pto resistance gene." 174: 1604-1611 (1992)). Thus, a homolog of *avrPto* was identified in avirulent *P. s. tomato* strain DC3000 based on DNA blot hybridization (Ronald et al., "The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the Pto resistance gene." 174: 1604-1611 (1992)). Gene replacement strains in which the *avrPto* reading frame was deleted were constructed in strains JL1065 and DC3000. Surprisingly, both mutant strains were still recognized by *Pto*-expressing tomato leaves (Ronald et al., "The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the Pto resistance gene." 174: 1604-1611 (1992)). A later study found that a tomato line carrying a CaMV 35S::*Pto* transgene (and not a sibling line without *Pto*) is resistant to the *avrPto*ΔDC3000 deletion strain. These results implied that strains DC3000 and JL1065 carry additional avirulence proteins that are recognized specifically by Pto.

[0011] In recent years, evidence has accumulated that effector proteins can interfere with host defense responses. In a breakthrough study, Jackson et al., "Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native plasmid in the bean pathogen Pseudomonas syringae pathovar phaseolicola." *Proc Natl Acad Sci USA*, 96: 10875-10880 (1999) demonstrated that VirPphA allows *P. s.* pv. *phaseolicola* to evade HR-based immunity in bean. Other *P. s.* pv. *phaseolicola* effectors also allow the pathogen to avoid triggering host immunity, including AvrPphC and AvrPphF (Tsiamis et al., "Cultivar-specific avirulence and virulence functions assigned to avrPphF in Pseudomonas syringae pv. phaseolicola, the cause of bean halo-blight disease." *Embo J*, 19: 3204-3214 (2000)). Additionally, in the *P. s.* pv. *maculicola*-Arabidopsis pathosystem, interference has been observed with the effector proteins AvrRpt2 and AvrRpm1 and the HR initiated by the R proteins RPS2 and RPM1, respectively (Reuber et al, "Isolation of arabidopsis genes that differentiate

between resistance responses mediated by the RPS2 and RPM1 disease resistance genes." *Plant Cell*, 8: 241-249 (1996); Ritter et al., "Interference between two specific pathogen recognition events mediated by distinct plant disease resistance genes." *Plant Cell*, 8: 251-257 (1996)). These findings suggest that for some effector proteins virulence activity can be dominant over avirulence activity. Although the phenomenon of effector-mediated evasion of plant immunity has been well documented, the molecular basis of this activity has remained mysterious. Several hypotheses have been proposed to explain how some effector proteins (such as VirPphA, AvrPphC and AvrPphF) prevent a host from detecting a pathogen, including: i) inhibition of *avr* gene expression; ii) blocking of Avr protein secretion or translocation; iii) interference with Avr/R protein recognition inside the plant cell; or iv) suppression of HR or disease resistance signaling downstream of Avr recognition (Jackson et al., "Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native plasmid in the bean pathogen Pseudomonas syringae pathovar phaseolicola." *Proc Natl Acad Sci USA*, 96: 10875-10880 (1999); Tsiamis et al., "Cultivar-specific avirulence and virulence functions assigned to avrPphF in Pseudomonas syringae pv. phaseolicola, the cause of bean halo-blight disease." *Embo J*, 19: 3204-3214 (2000)). Specific support, however, for any one of these hypotheses has not been reported.

[0012] The present invention is directed to overcoming these and other deficiencies in the art.

SUMMARY OF THE INVENTION

[0013] The present invention relates to a bacterial effector protein which inhibits programmed cell death in eukaryotes.

[0014] The present invention also relates to a nucleic acid molecule encoding a bacterial effector protein which inhibits programmed cell death in eukaryotes.

[0015] Another aspect of the present invention pertains to host cells, transgenic plants, and transgenic plant seeds containing a nucleic acid molecule

encoding a bacterial effector protein which inhibits programmed cell death in eukaryotes.

[0016] The present invention is also directed to a method of suppressing programmed cell death in eukaryotes. This method involves transforming a eukaryote with a nucleic acid encoding a bacterial effector protein which inhibits programmed cell death in eukaryotes. The eukaryote is then grown under conditions effective to suppress programmed cell death in the eukaryote.

[0017] A further aspect of the present invention relates to a method of delaying senescence in plants. This method involves transforming a plant with a nucleic acid encoding a bacterial effector protein which inhibits programmed cell death in eukaryotes. The plant is then grown under conditions effective to delay senescence in the plant.

[0018] Yet another aspect of the present invention relates to a method of increasing protein expression in plants. This method involves transforming a plant with a nucleic acid encoding a first bacterial effector protein which inhibits programmed cell death in eukaryotes and a second protein which is toxic to plants. The plant is grown under conditions effective to increase expression of the second protein in the plant.

[0019] Another aspect of the present invention pertains to expression vectors, transgenic plants, and transgenic plant seeds containing a nucleic acid construct having a nucleic acid molecule encoding a first bacterial effector protein of the present invention coupled to a nucleic acid molecule producing a second protein toxic to eukaryotes.

[0020] Yet another aspect of the present invention relates to a method of stabilizing a transgenic plant producing a protein toxic to plants. This method involves providing a transgenic plant transduced with a nucleic acid molecule encoding a first bacterial effector protein which inhibits programmed cell death in eukaryotes and a nucleic acid molecule producing a protein toxic to plants. The plant is then grown under conditions effective to stabilize the plant.

[0021] A further aspect of the present invention relates to a method of treating a subject for conditions mediated by programmed cell death. This method involves administering to the subject a bacterial effector protein which inhibits

programmed cell death under conditions effective to treat the condition mediated by programmed cell death.

[0022] The bacterial effector proteins of the present invention can be used to inhibit programmed cell death in eukaryotes. In particular, AvrPtoB will be a useful tool to dissect the molecular basis of plant R protein programmed cell death signaling, which presently is poorly understood. AvrPtoB anti-PCD activity may also have biotechnical applications. For example, AvrPtoB may allow efficient transgenic expression of proteins that otherwise elicit host PCD or may function to alter PCD-dependent plant developmental processes, such as senescence. Increased understanding of the complex basis of effector-mediated PCD inhibition and host mechanisms that guard against PCD inhibition, should lead to further novel insights into the molecular basis of plant immunity and disease.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] Figures 1A-C show AvrPtoB-mediated inhibition of PCD in *N. benthamiana*. Figure 1A shows proteins co-expressed in *N. benthamiana* using *Agrobacterium-mediated* transient expression. Leaves were agroinfiltrated within the marked circles and photos were taken 7 days after agroinfiltration. Figure 1B shows AvrPtoB suppressing PCD initiated by AvrPto/Pto recognition in *N. benthamiana*. The *N. benthamaniana* leaf was agroinfiltrated with AvrPto and Pto and left to dry. On the left hand side, AvrPtoB was agroinfiltrated, and on the right hand side, a vector control was agroinfiltrated. After 7 days, an island of PCD suppressed tissue was observed in AvrPtoB expressing cells. Figure 1C is an immunoblot analysis of AvrPto:HA, AvrPtoB:HA and Pto:HA co-expression in *N. benthamiana*. Lane 1: AvrPto, AvrPtoB, Pto; 2, AvrPtoB; 3, Pto; 4, AvrPto.

[0024] Figures 2A-B show AvrPtoB suppresses oxidative and heat stress-induced cell death in yeast. Figure 2A shows AvrPtoB protecting *S. cerevisiae* strain EGY48 from 3 mM H_2O_2 -induced PCD. The agar plates show increased survival of yeast cells expressing AvrPtoB as compared to the wild type after treatment with 3 mM H_2O_2 . Figure 2B shows AvrPtoB protecting yeast from cell death triggered by: 1) 3 mM H_2O_2, 2) 5 mM H_2O_2, 3) 5 mM menadione, 4) 10 mM menadione, 5) heat shock at 50 °C, and 6) heat shock at 50°C with a 37°C pre-

treatment. White bars represent wild type yeast and black bars represent AvrPtoB expressing yeast. Error bars show the standard deviation about the mean for three trials.

[0025] Figures 3A-B show structural analysis of AvrPtoB recognition and anti-PCD activity. Figure 3A is a schematic representation of AvrPtoB truncations examined in this study and yeast two-hybrid analysis of physical interactions between AvrPtoB truncations and the Pto R protein. AvrPtoB truncations were cloned as bait fusions and tested against a Pto prey fusion. Constructs shaded black interacted strongly with Pto. Figure 3B shows *in planta* transient expression of AvrPtoB truncations in tomato. RG-PtoR, RG-pto11 and RG-prf3 are isogenic tomato lines with the *L. pimpenillifolium* Pto haplotype and genotypes as indicated. RG-ptoS is a near-isogenic line with the *L. esculentum* Pto haplotype. *Note: a late-onset weak cell death phenotype was observed with Δ6 expression in RG-ptoS. + = cell death, - = no response.

[0026] Figures 4A-C show recognition and anti-PCD activity of AvrPtoB truncations in *N. benthamiana*. Figure 4A shows full length and truncated AvrPtoB constructs were transiently expressed: i) with AvrPto + Pto to test for anti-PCD activity, ii) with Pto to test for Pto-mediated PCD, and iii) alone to test for *Rsb*-mediated PCD. Protein expression of each truncation is established by an observable phenotype. Figure 4B shows epistasis experiments examining the molecular basis of Δ6/Pto- and Δ7/Pto-initiated PCD and Figure 4C shows Δ6-initiated PCD. Intact AvrPtoB suppressed PCD initiated by Δ6/Pto, Δ7/Pto, and Δ6, suggesting an intermolecular mechanism of anti-PCD activity. Photos were taken 7 days after agroinfiltration.

[0027] Figures 5A-B show *P. s.* pv. *tomato* DC3000 chromosomal mutants of *avrPtoB* and disease responses of inoculated tomato plants. Figure 5A is a schematic representation of *avrPtoB* chromosomal mutations in *P. s.* pv. *tomato* DC3000, generated by insertion of the 6 kb pKnockout plasmid. Amino acid numbers correspond to the amino-acid residue where the expressed mutant protein is interrupted by the insertion. Figure 5B shows disease responses of tomato plants inoculated with DC3000:mut mutants. Note that only DC3000::mut5 triggers immunity in RG-pto11 plants and this is the only mutant that expresses

AvrPtoB with the *Rsb* triggering domain described in the text. The immunity observed in RG-PtoR plants is likely the result of AvrPto recognition. I = Immunity, D = Disease.

[0028] Figures 6A-B show AvrPtoB induces plant susceptibility to *P. s.* pv. *tomato* DC3000 infection. Figure 6A shows disease symptoms or host immunity on tomato leaves 6 days after inoculation with indicated bacterial strains. Mutant DC3000::mut5 triggers immunity in RG-pto11 and expression of AvrPtoB *in trans* restores DC3000::mut5 pathogenicity. pDSK519 is a broad host range plasmid. I = Immunity; D = Disease. Figure 6B shows bacterial growth in leaves over a period of 6 days as measured by the number of colony forming units (cfu) per cm^2 of leaf tissue. Errors bars represent the standard deviation of bacterial counts.

[0029] Figures 7A-E show AvrPtoB, AvrPtoBT1, AvrPtoBT23, and AvrPtoBJL1065 amino acid sequences aligned by Clustal V method provided by DNAStar software (DNAStar, Inc., Madison, WI). Identical amino acids among sequences are shown in black boxes.

[0030] Figure 8 shows a model for AvrPtoB recognition and PCD inhibition in tomato. The modular structure of AvrPtoB is depicted with the Pto-recognized N-terminal module shown as a brown circle, the anti-PCD C-terminal module shown as a red octagon, and the region recognized by *Rsb* shown as a blue connecting line. The black box represents an unknown factor predicted to act with Pto to suppress AvrPtoB anti-PCD function. *Rsb*-mediated cell death and immunity only occurs in the presence of the Δ6 truncation and the absence of Pto and intact AvrPtoB. Note: the gene(s) controlling the *Rsb* phenotype has not been identified; therefore, *Rsb* is presented in this model as a hypothetical R protein.

[0031] Figures 9A-C show interaction of *Pseudomonas* effector protein AvrPtoB with the Pto kinase in the yeast two-hybrid system and features of the AvrPtoB gene. Figure 9A shows a test for specificity of AvrPtoB and AvrPto interaction with Pto family proteins and Pti1 kinase in the LexA yeast two-hybrid system. The *avrPtoB* (PtiDC1Δ70) and *avrPto* genes were cloned into the prey vector pJG4-5 and the Pto, Pti1, and Bicoid genes were cloned into the bait vector pEG202. The constructs were transformed into yeast strain EGY48 carrying a *lacZ* reporter gene and the cells were plated onto medium containing X-gal. Dark

blue color indicates interaction. Figure 9B shows the nucleotide sequence and corresponding encoded amino acid sequence of the *avrPtoB* gene (GenBank Acc. No. AY074795). The region upstream of the putative start codon shows the Hrp box cis element and the entire open reading frame of *avrPtoB*. The amino acids of the AvrPtoB protein are given in single letter code. Figure 9C shows database search results using the *avrPtoB* gene. The *avrPtoB* genomic DNA sequence was used to search the National Center for Biotechnology Information sequence database using the BlastX algorithm. *VirPphA* was the gene in the database with the greatest similarity to *avrPtoB* with an E value of e^{-140}. Amino acid alignments of AvrPtoB (SEQ ID NO:2) and VirPphA (SEQ ID NO: 52) reveal substantial amino acid sequence conservation across both predicted proteins, with 52% amino acid identity and 63% amino acid similarity.

[0032] Figures 10A-C show interaction of AvrPtoB and AvrPto with the same variant forms of the Pto kinase. Figure 10A shows interactions of Pto-Fen chimeric proteins (Tang et al., "The avirulence protein AvrPto physically interacts with the Pto kinase." *Science* 274: 2060-2063 (1996), which is hereby incorporated by reference in its entirety) with AvrPtoB and AvrPto in the LexA yeast two-hybrid system. The diagram depicts Pto (black regions) and Fen (white regions) chimeric proteins. EGY48 yeast cells containing the Pto-Fen chimeric proteins in bait vector pEG202, AvrPtoB or AvrPto in prey vector pJG4-5, and the *lacZ* reporter gene were grown on medium containing *X-gal*. Equal expression of each chimeric protein was verified by Western blot (Tang et al., "The avirulence protein AvrPto physically interacts with the Pto kinase." *Science* 274: 2060-2063 (1996), which is hereby incorporated by reference in its entirety). Figure 10B shows interaction of the internal region of Pto (Frederick et al., "Recognition specificity for the bacterial avirulence protein AvrPto is determined by Thr-204 in the activation loop of the tomato Pto kinase." *Molecular Cell.* 2: 241-245 (1998), which is hereby incorporated by reference in its entirety) with AvrPtoB or AvrPto in the LexA yeast two-hybrid system. Chimeric proteins FPB, FPB2, FPB3 and FPB4 contain the amino acids from Pto (black regions) or from Fen (white regions). Numbers corresponding to amino acid positions in Pto are indicated. Equal expression of Pto-Fen chimeric proteins was verified by Western blot (Frederick et al., "Recognition specificity for the bacterial avirulence protein

AvrPto is determined by Thr-204 in the activation loop of the tomato Pto kinase." *Molecular Cell.* 2: 241-245 (1998), which is hereby incorporated by reference in its entirety). Figure 10C shows the effect of amino acid substitutions in Pto/Fen kinase subdomain VIII on the interaction with AvrPtoB and AvrPto in the yeast two-hybrid system. Portions of proteins, and individual amino acids, derived from Pto (in black) or Fen (in white) are shown. Unboxed amino acids are identical in both kinases. The numbering of amino acids and designation of substitutions correspond to the Pto sequence (Martin et al., "Map-based cloning of a protein kinase gene conferring disease resistance in tomato." *Science*, 262: 1432-1436 (1993), which is hereby incorporated by reference in its entirety).

[0033] Figure 11 shows DNA sequences with similarity to *avrPtoB* are present in diverse bacterial plant pathogens. DNA was isolated from the *Pseudomonas*, *Xanthomonas*, or *Erwinia* strains indicated and analyzed on a gel blot using a radiolabeled *avrPtoB* gene probe. Stringency of the final wash was 0.1X SSC, 0.1% SDS.

[0034] Figures 12A-C show AvrPtoB is secreted via the *Pseudomonas* type III secretion system and elicits a *Pto*- and *Prf*-specific HR in tomato leaves. Figure 12A shows elicitation of a *Pto*- and *Prf*-specific HR in tomato leaves by a *P. fluorescens* strain expressing a type III secretion system and *avrPtoB*. Tomato leaves of the indicated genotypes were syringe-infiltrated with $1x10^7$ cfu/mL of *P. fluorescens* (pHIR11; Hrp+) carrying *avrPtoB* on the wide host range vector pDSK519. The HR appeared within 24 hr only in RG-PtoR leaves (see arrow; some necrosis due to wounding with the syringe is visible on other leaves). Infiltration of $1x10^7$ cfu/mL of *P. fluorescens* (pHIR11; Hrp+) with pDSK519 alone elicited no response in any leaf genotypes. Photographs were taken 4 days after infiltration. Figure 12B shows elicitation of a *Pto*- and *Prf*-specific HR in tomato leaves upon expression of an *avrPtoB* transgene directly in plant cells. A suspension of *Agrobacterium* strain GV2260 (OD_{600} = 0.06) carrying a binary vector with an *avrPtoB* transgene expressed by the CaMV 35S promoter was infiltrated into leaves of the indicated genotypes. The HR appeared within 18 hr only in RG-PtoR leaves (see arrow). Infiltration of *Agrobacterium* carrying an empty binary vector elicited no response in any leaf genotypes. Photographs were taken 4 days after infiltration. Figure 12C shows co-expression of *Pto* and

avrPtoB transgenes directly in *pto* mutant leaf cells elicits the HR. A mixture of suspensions of *Agrobacterium* strain GV2260 (OD_{600} = 0.06) carrying a binary vector with an *avrPtoB* or *Pto* transgene transcribed by the CaMV 35S promoter was infiltrated into leaves of RG-pto11. The HR appeared within 24 hr only in leaves expressing both *Pto* and *avrPtoB* (arrow). Infiltration of *Agrobacterium* carrying an empty binary vector elicited no response in any leaf genotypes. Photographs were taken 4 days after infiltration.

[0035] Figures 13A-B show that AvrPtoB and AvrPto share discrete regions in common and subregion II is conserved among diverse bacterial effector proteins. Figure 13A shows how amino acid sequences of AvrPtoB (SEQ ID NO:2)(top) and AvrPto (SEQ ID NO:53)(bottom) were aligned using DNAStar software and visually; dashes indicate gaps introduced to optimize the alignment. Nine subregions which contain identical amino acids are shown in boxes. The glycine residue present in the myristylation motif of AvrPto is underlined. Dots indicate residues of AvrPto in which substitutions cause loss of Pto interaction in yeast two-hybrid system and HR in *Pto*-expressing tomato leaves (Shan et al., "The *Pseudomonas* AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane." *Plant Cell* 12: 2323-2337 (2000), which is hereby incorporated by referenc in its entirety). The arrow in subregion III indicates the most N-terminal truncated AvrPtoB protein (Δ121) that still interacts with Pto in the two-hybrid system. The arrow in subregion VIII indicates the most C-terminal truncated form of AvrPto (Δ40) that still interacts with Pto in the two-hybrid system (Chang et al., "Functional studies of the bacterial avirulence protein AvrPto by mutational analysis." *Mol. Plant-Microbe Interact.* 14: 451-459 (2001), which is hereby incorporated by reference in its entirety). The 'GINP' motif is boxed (located in subregion V) and the substitutions which were made in this region are shown above AvrPtoB. Figure 13B shows alignment of part of subregion III in AvrPtoB and AvrPto that shares similar residues with diverse effector proteins from other bacterial phytopathogens. A consensus (SEQ ID NO:54) is also shown at the top. The amino acid position of the region in each effector protein is indicated. Origin of the effectors is: *P. s. tomato* strain (AvrPto, AvrPtoB, AvrRpt2), *P. s. glycinea* (AvrB), *P. s. phaseolicola* (VirPphA, AvrPphF), *P. s. pisi* (AvrRps4, AvrPpiB),

Xanthomonas campestris pv. *vesicatoria* (AvrBs1, AvrBsT), and *X. oryzae* pv. *oryzae* (AvrXa10).

[0036] Figure 14 shows a motif shared by AvrPtoB and AvrPto is required for interaction with the Pto kinase. Amino acid substitutions in and near subregion V were created in AvrPtoB and the mutant proteins were tested for interaction with the Pto kinase in the LexA yeast two-hybrid system. Degree of *lacZ* reporter gene activation was determined by measuring relative units of β - galactosidase activity in yeast strains expressing the mutant proteins and Pto (as in Frederick et al., "Recognition specificity for the bacterial avirulence protein AvrPto is determined by Thr-204 in the activation loop of the tomato Pto kinase." *Molecular Cell.* 2: 241-245 (1998), which is hereby incorporated by reference in its entirety). The β - galactosidase activity data are the means (gray boxes) and standard errors (error bars) of duplicate experiments each with three independent colonies per construct.

[0037] Figure 15 shows tomato leaves (variety Rio Grande-PtoR) expressing Pto that were inoculated with the *Pseudomonas syringae* pv. *tomato* strain PT11 or derivatives of this strain. In the top right panel PT11 expresses avrPtoB from an introduced plasmid (PT11(avrPtoB)). In the bottom panels, PT11 expresses mutant versions of avrPtoB from an introduced plasmid (the mutations each cause a substitution of one amino acid: isoleucine to threonine at amino acid position 326 or glycine to alanine at amino acid position 333. The conclusion from the experiments is that introduction of avrPtoB into the normally disease-causing strain PT11 allows it to now be recognized by Pto. Mutation of a residue at position 326 abolishes this recognition while a mutation at position 333 does not affect it. This data supports a role for subdomain V in the recognition of AvrPtoB by the Pto kinase (as also supported by data presented in Table 2).

DETAILED DESCRIPTION OF THE INVENTION

[0038] The present invention relates to a bacterial effector protein which inhibits programmed cell death in eukaryotes.

[0039] The present invention also relates to a nucleic acid molecule encoding a bacterial effector protein which inhibits programmed cell death in eukaryotes.

[0040] In the first aspect of the present invention, the bacterial effector protein is identified herein as *avrPtoB (PstDC3000)* and is encoded by a nucleic acid molecule having a nucleotide sequence of SEQ ID NO:1 as follows:

```
atggcgggta tcaatagagc gggaccatcg ggcgcttatt ttgttggcca cacagacccc 60
gagccagtat cggggcaagc acacggatcc ggcagcggcg ccagctcctc gaacagtccg 120
caggttcagc cgcgaccctc gaatactccc ccgtcgaacg cgcccgcacc gccgccaacc 180
ggacgtgaga ggctttcacg atccacggcg ctgtcgcgcc aaaccaggga gtggctggag 240
cagggtatgc ctacagcgga ggatgccagc gtgcgtcgta ggccacaggt gactgccgat 300
gccgcaacgc cgcgtgcaga ggcaagacgc acgccggagg caactgccga tgccagcgca 360
ccgcgtagag gggcggttgc acacgccaac agtatcgttc agcaattggt cagtgagggc 420
gctgatattt cgcatactcg taacatgctc cgcaatgcaa tgaatggcga cgcagtcgct 480
ttttctcgag tagaacagaa catatttcgc cagcatttcc cgaacatgcc catgcatgga 540
atcagccgag attcggaact cgctatcgag ctccgtgggg cgcttcgtcg agcggttcac 600
caacaggcgg cgtcagcgcc agtgaggtcg cccacgccaa caccggccag ccctgcggca 660
tcatcatcgg gcagcagtca gcgttcttta tttggacggt ttgcccgttt gatggcgcca 720
aaccagggac ggtcgtcgaa cactgccgcc tctcagacgc cggtcgacag gagcccgcca 780
cgcgtcaacc aaagacccat acgcgtcgac agggctgcga tgcgtaatcg tggcaatgac 840
gaggcggacg ccgcgctgcg ggggttagta caacaggggg tcaatttaga gcacctgcgc 900
acggcccttg aaagacatgt aatgcagcgc ctccctatcc ccctcgatat aggcagcgcg 960
ttgcagaatg tgggaattaa cccaagtatc gacttggggg aaagccttgt gcaacatccc 1020
ctgctgaatt tgaatgtagc gttgaatcgc atgctggggc tgcgtcccag cgctgaaaga 1080
gcgcctcgtc cagccgtccc cgtggctccc gcgaccgcct ccaggcgacc ggatggtacg 1140
cgtgcaacac gattgcgggt gatgccggag cgggaggatt acgaaaataa tgtggcttat 1200
ggagtgcgct tgcttaacct gaacccgggg gtgggggtaa ggcaggctgt tgcggccttt 1260
gtaaccgacc gggctgagcg gccagcagtg gtggctaata tccgggcagc cctggaccct 1320
atcgcgtcac aattcagtca gctgcgcaca atttcgaagg ccgatgctga atctgaagag 1380
ctgggtttta aggatgcggc agatcatcac acggatgacg tgacgcactg tctttttggc 1440
ggagaattgt cgctgagtaa tccggatcag caggtgatcg gtttggcggg taatccgacg 1500
gacacgtcgc agccttacag ccaagaggga ataaggacc tggcgttcat ggatatgaaa 1560
aaacttgccc aattcctcgc aggcaagcct gagcatccga tgaccagaga aacgcttaac 1620
gccgaaaata tcgccaagta tgcttttaga atagtcccct ga                    1662
```

[0041] The nucleic acid sequence corresponding to SEQ ID NO:1 encodes a bacterial effector protein identified herein as AvrPtoB (Pst DC3000), which has a deduced amino acid sequence corresponding to SEQ ID NO:2 as follows:

```
Met Ala Gly Ile Asn Arg Ala Gly Pro Ser Gly Ala Tyr Phe Val Gly
 1               5                  10                  15

His Thr Asp Pro Glu Pro Val Ser Gly Gln Ala His Gly Ser Gly Ser
            20                  25                  30

Gly Ala Ser Ser Ser Asn Ser Pro Gln Val Gln Pro Arg Pro Ser Asn
        35                  40                  45

Thr Pro Pro Ser Asn Ala Pro Ala Pro Pro Pro Thr Gly Arg Glu Arg
    50                  55                  60

Leu Ser Arg Ser Thr Ala Leu Ser Arg Gln Thr Arg Glu Trp Leu Glu
65                  70                  75                  80
```

Gln Gly Met Pro Thr Ala Glu Asp Ala Ser Val Arg Arg Arg Pro Gln
85 90 95

Val Thr Ala Asp Ala Ala Thr Pro Arg Ala Glu Ala Arg Arg Thr Pro
100 105 110

Glu Ala Thr Ala Asp Ala Ser Ala Pro Arg Arg Gly Ala Val Ala His
115 120 125

Ala Asn Ser Ile Val Gln Gln Leu Val Ser Glu Gly Ala Asp Ile Ser
130 135 140

His Thr Arg Asn Met Leu Arg Asn Ala Met Asn Gly Asp Ala Val Ala
145 150 155 160

Phe Ser Arg Val Glu Gln Asn Ile Phe Arg Gln His Phe Pro Asn Met
165 170 175

Pro Met His Gly Ile Ser Arg Asp Ser Glu Leu Ala Ile Glu Leu Arg
180 185 190

Gly Ala Leu Arg Arg Ala Val His Gln Gln Ala Ala Ser Ala Pro Val
195 200 205

Arg Ser Pro Thr Pro Thr Pro Ala Ser Pro Ala Ala Ser Ser Ser Gly
210 215 220

Ser Ser Gln Arg Ser Leu Phe Gly Arg Phe Ala Arg Leu Met Ala Pro
225 230 235 240

Asn Gln Gly Arg Ser Ser Asn Thr Ala Ala Ser Gln Thr Pro Val Asp
245 250 255

Arg Ser Pro Pro Arg Val Asn Gln Arg Pro Ile Arg Val Asp Arg Ala
260 265 270

Ala Met Arg Asn Arg Gly Asn Asp Glu Ala Asp Ala Ala Leu Arg Gly
275 280 285

Leu Val Gln Gln Gly Val Asn Leu Glu His Leu Arg Thr Ala Leu Glu
290 295 300

Arg His Val Met Gln Arg Leu Pro Ile Pro Leu Asp Ile Gly Ser Ala
305 310 315 320

Leu Gln Asn Val Gly Ile Asn Pro Ser Ile Asp Leu Gly Glu Ser Leu
325 330 335

Val Gln His Pro Leu Leu Asn Leu Asn Val Ala Leu Asn Arg Met Leu
340 345 350

Gly Leu Arg Pro Ser Ala Glu Arg Ala Pro Arg Pro Ala Val Pro Val
355 360 365

Ala Pro Ala Thr Ala Ser Arg Arg Pro Asp Gly Thr Arg Ala Thr Arg

```
    370                 375                 380

Leu Arg Val Met Pro Glu Arg Glu Asp Tyr Glu Asn Asn Val Ala Tyr
385                 390                 395                 400

Gly Val Arg Leu Leu Asn Leu Asn Pro Gly Val Gly Val Arg Gln Ala
                405                 410                 415

Val Ala Ala Phe Val Thr Asp Arg Ala Glu Arg Pro Ala Val Val Ala
            420                 425                 430

Asn Ile Arg Ala Ala Leu Asp Pro Ile Ala Ser Gln Phe Ser Gln Leu
        435                 440                 445

Arg Thr Ile Ser Lys Ala Asp Ala Glu Ser Glu Glu Leu Gly Phe Lys
    450                 455                 460

Asp Ala Ala Asp His His Thr Asp Asp Val Thr His Cys Leu Phe Gly
465                 470                 475                 480

Gly Glu Leu Ser Leu Ser Asn Pro Asp Gln Gln Val Ile Gly Leu Ala
                485                 490                 495

Gly Asn Pro Thr Asp Thr Ser Gln Pro Tyr Ser Gln Glu Gly Asn Lys
            500                 505                 510

Asp Leu Ala Phe Met Asp Met Lys Lys Leu Ala Gln Phe Leu Ala Gly
        515                 520                 525

Lys Pro Glu His Pro Met Thr Arg Glu Thr Leu Asn Ala Glu Asn Ile
    530                 535                 540

Ala Lys Tyr Ala Phe Arg Ile Val Pro
545                 550
```

This bacterial effector protein has a molecular mass from 55-65 kDa.

[0042] In another aspect of the present invention, another suitable bacterial effector protein of the present invention is identified herein as *avrPtoB (H Pst T1)* and is encoded by a nucleic acid molecule having a nucleotide sequence of SEQ ID NO:3 as follows:

```
atggcgggta tcaatggagc gggaccatcg ggcgcttatt ttgttggcca cacagacccc 60
gagccagcat cgggggcgc acacggatcc agcagtggcg cgagatcctc gaacagtccg 120
cgcttgccgg cgcctccgga tgcacccgcg tcgcaggcgc gagatcgacg cgaaatgctt 180
ttgcgagcca ggccgctgtc gcgccaaacc agggagtggg tggcgcaggg tatgccgcca 240
acggcggagg ctggagtgcc catcaggccg caggagtctg ccgaggctgc agcgccgcag 300
gcacgtgcag aggaaagaca cacgccggag gctgatgcag cagcgtcgca tgtacgcaca 360
gagggaggac gcacaccgca ggcgcttgcc ggtacctccc cacgccacac aggtgcggtg 420
ccacacgcca atagaattgt tcaacaattg gttgacgcgg gcgctgatct tgccggtatt 480
aacaccatga ttgacaatgc catgcgtcgc cacgcgatag ctcttccttc tcgaacagta 540
cagagtattt tgatcgagca tttccctcac ctgctagcgg gtgaactcat tagtggctca 600
gagctcgcta ccgcgttccg tgcggctctc cgtcgggagg ttcgccaaca ggaggcgtca 660
gccccccaa gaacagcagc gcggtcctcc gtaaggacgc cggagcggtc gacggtgccg 720
cccacttcta cggaatcatc atcgggcagc aaccagcgta cgttattagg gcggttcgcc 780
```

```
gggttgatga cgcctaatca gagacgtccg tcgagcgctt cgaacgcgtc tgcctctcaa 840
aggcctgtag acagaagccc gccacgcgta aaccaggtac ccacaggcgc taacagggtt 900
gtgatgcgta atcatggtaa taacgaggcc gacgccgcgc tgcaaggatt ggctcagcag 960
ggggttgata tggaggacct gcgcgccgcg cttgaaagac atatattgca tcgccgcccg 1020
atccccatgg atatagcgta cgccttgcag ggtgtgggca ttgcgccaag tatcgatacg 1080
ggagagagcc ttatggaaaa cccgctgatg aatttgagtg ttgcgctgca ccgcgcacta 1140
gggcctcgtc ccgctcgtgc tcaagcgcct cgtccagccg ttccggtggc tcccgcgacc 1200
gtctccaggc gaccagatag cgcgcgtgcc acaagattgc aggtaatacc ggcgcgggag 1260
gattacgaaa ataatgtggc ctacggagtg cgcttgctga gcctgaatcc gggcgcgggg 1320
gtcagggaga ctgttgcggc ctttgtaaac aaccgttacg agcggcaggc ggttgttgcc 1380
gacatacgcg cagccctaaa tttatctaaa caattcaata agttgcgtac ggtctctaag 1440
gccgatgctg cctccaataa accgggcttc aaggatgcgg cggaccaccc agacgacgcg 1500
acgcaatgcc tttttggtga agaattgtcg ctgaccagtt cggatcagca ggtgatcggc 1560
ctggcaggta aggcaacgga catgtcggag tcttacagcc gagaggcaaa taaggacctg 1620
gtgttcatgg atatgaaaaa acttgcccaa ttcctcgcag gcaagcctga gcatccgatg 1680
accagagaaa cgcttaacgc cgaaaatatc gccaagtatg cttttagaat agtcccctga 1740
```

[0043] The nucleic acid sequence corresponding to SEQ ID NO:3 encodes a bacterial effector protein identified herein as AvrPtoB (H Pst T1), which has a deduced amino acid sequence corresponding to SEQ ID NO:4 as follows:

```
Met Ala Gly Ile Asn Gly Ala Gly Pro Ser Gly Ala Tyr Phe Val Gly
 1               5                  10                  15

His Thr Asp Pro Glu Pro Ala Ser Gly Gly Ala His Gly Ser Ser Ser
            20                  25                  30

Gly Ala Arg Ser Ser Asn Ser Pro Arg Leu Pro Ala Pro Pro Asp Ala
        35                  40                  45

Pro Ala Ser Gln Ala Arg Asp Arg Arg Glu Met Leu Leu Arg Ala Arg
    50                  55                  60

Pro Leu Ser Arg Gln Thr Arg Glu Trp Val Ala Gln Gly Met Pro Pro
65                  70                  75                  80

Thr Ala Glu Ala Gly Val Pro Ile Arg Pro Gln Glu Ser Ala Glu Ala
                85                  90                  95

Ala Ala Pro Gln Ala Arg Ala Glu Glu Arg His Thr Pro Glu Ala Asp
            100                 105                 110

Ala Ala Ala Ser His Val Arg Thr Glu Gly Gly Arg Thr Pro Gln Ala
        115                 120                 125

Leu Ala Gly Thr Ser Pro Arg His Thr Gly Ala Val Pro His Ala Asn
    130                 135                 140

Arg Ile Val Gln Gln Leu Val Asp Ala Gly Ala Asp Leu Ala Gly Ile
145                 150                 155                 160

Asn Thr Met Ile Asp Asn Ala Met Arg Arg His Ala Ile Ala Leu Pro
                165                 170                 175

Ser Arg Thr Val Gln Ser Ile Leu Ile Glu His Phe Pro His Leu Leu
            180                 185                 190
```

Ala Gly Glu Leu Ile Ser Gly Ser Glu Leu Ala Thr Ala Phe Arg Ala
195 200 205

Ala Leu Arg Arg Glu Val Arg Gln Gln Glu Ala Ser Ala Pro Pro Arg
210 215 220

Thr Ala Ala Arg Ser Ser Val Arg Thr Pro Glu Arg Ser Thr Val Pro
225 230 235 240

Pro Thr Ser Thr Glu Ser Ser Ser Gly Ser Asn Gln Arg Thr Leu Leu
245 250 255

Gly Arg Phe Ala Gly Leu Met Thr Pro Asn Gln Arg Arg Pro Ser Ser
260 265 270

Ala Ser Asn Ala Ser Ala Ser Gln Arg Pro Val Asp Arg Ser Pro Pro
275 280 285

Arg Val Asn Gln Val Pro Thr Gly Ala Asn Arg Val Val Met Arg Asn
290 295 300

His Gly Asn Asn Glu Ala Asp Ala Ala Leu Gln Gly Leu Ala Gln Gln
305 310 315 320

Gly Val Asp Met Glu Asp Leu Arg Ala Ala Leu Glu Arg His Ile Leu
325 330 335

His Arg Arg Pro Ile Pro Met Asp Ile Ala Tyr Ala Leu Gln Gly Val
340 345 350

Gly Ile Ala Pro Ser Ile Asp Thr Gly Glu Ser Leu Met Glu Asn Pro
355 360 365

Leu Met Asn Leu Ser Val Ala Leu His Arg Ala Leu Gly Pro Arg Pro
370 375 380

Ala Arg Ala Gln Ala Pro Arg Pro Ala Val Pro Val Ala Pro Ala Thr
385 390 395 400

Val Ser Arg Arg Pro Asp Ser Ala Arg Ala Thr Arg Leu Gln Val Ile
405 410 415

Pro Ala Arg Glu Asp Tyr Glu Asn Asn Val Ala Tyr Gly Val Arg Leu
420 425 430

Leu Ser Leu Asn Pro Gly Ala Gly Val Arg Glu Thr Val Ala Ala Phe
435 440 445

Val Asn Asn Arg Tyr Glu Arg Gln Ala Val Val Ala Asp Ile Arg Ala
450 455 460

Ala Leu Asn Leu Ser Lys Gln Phe Asn Lys Leu Arg Thr Val Ser Lys
465 470 475 480

Ala Asp Ala Ala Ser Asn Lys Pro Gly Phe Lys Asp Ala Ala Asp His
485 490 495

```
Pro Asp Asp Ala Thr Gln Cys Leu Phe Gly Glu Glu Leu Ser Leu Thr
            500                 505                 510

Ser Ser Asp Gln Gln Val Ile Gly Leu Ala Gly Lys Ala Thr Asp Met
        515                 520                 525

Ser Glu Ser Tyr Ser Arg Glu Ala Asn Lys Asp Leu Val Phe Met Asp
    530                 535                 540

Met Lys Lys Leu Ala Gln Phe Leu Ala Gly Lys Pro Glu His Pro Met
545                 550                 555                 560

Thr Arg Glu Thr Leu Asn Ala Glu Asn Ile Ala Lys Tyr Ala Phe Arg
                565                 570                 575

Ile Val Pro
```

This bacterial effector protein has a molecular mass from 55-65 kDa.

[0044] Another suitable bacterial effector protein of the present invention is identified herein as *avrPtoB (H Pst PT23)* and is encoded by a nucleic acid molecule having a nucleotide sequence of SEQ ID NO:5 as follows:

```
atggcgggta tcaatggagc gggaccatcg ggcgcttatt ttgttggcca cacagacccc 60
gagccagcat cgggggcgc acacggatcc agcagtggcg caagctcctc gaacagtccg 120
cgcttgccgg cgcctccgga tgcacccgcg tcgcaggcgc gagatcgacg cgaaatgctt 180
ttgcgagcca ggccgctgtc gcgccaaacc agggagtggg tggcgcaggg tatgccgcca 240
acggcggagg ctggagtgcc catcaggccg caggagtctg ccgaggctgc agcgccgcag 300
gcacgtgcag aggaaagaca cacgccggag gctgatgcag cagcgtcgca tgtacgcaca 360
gagggaggac gcacaccgca ggcgcttgcc ggtacctccc cacgccacac aggtgcggtg 420
ccacacgcca atagaattgt tcaacaattg gttgacgcgg gcgctgatct tgccggtatt 480
aacaccatga ttgacaatgc catgcgtcgc cacgcgatag ctcttccttc tcgaacagta 540
cagagtattt tgatcgagca tttccctcac ctgctagcgg gtgaactcat tagtggctca 600
gagctcgcta ccgcgttccg tgcggctctc cgtcgggagg ttcgccaaca ggaggcgtca 660
gccccccaa gaacaacagc gcggtcctcc gtaaggacgc cggagcggtc gacggtgccg 720
cccacttcta cggaatcatc atcgggcagc aaccagcgta cgttattagg gcggttcgcc 780
gggttgatga cgcctaatca gagacgtccg tcgagcgctt cgaacgcgtc tgcctctcaa 840
aggcctgtag acagaagccc gccacgcgta aaccaggtac ccacaggcgc taacagggtt 900
gtgatgcgta atcatggtaa taacgaggcc gacgccgcgc tgcaaggatt ggctcagcag 960
ggggttgata tggaggacct gcgcgccgcg cttgaaagac atatattgca tcgccgcccg 1020
atccccatgg atatagcgta cgccttgcag ggcgtgggca ttgcgccaag tatcgatacg 1080
ggagagagcc ttatggaaan cccgctgatg aatttgagtg ttgcgctgca ccgcgcacta 1140
gggcctcgtc ccgctcgtgc tcaagcgcct cgtccagccg ttccggtggc tcccgcgacc 1200
gtctccaggc gaccagatag cgcgcgtgcc acaagattgc aggtaatacc ggcgcgggag 1260
gattacgaaa ataatgtggc ctacggagtg cgcttgctga gcctgaatcc gggcgcgtgg 1320
gtcagggaga ctgttgcggc ctttgtaaac aaccgttacg agcggcaggc ggttgttgcc 1380
gacatacgcg cagccctaaa tttatctaaa caattcaata agttgcgtac ggtctctaag 1440
```

```
gccgatgctg cctccaataa accgggcttc aaggatctgg cggaccaccc agacgacgcg 1500
acgcaatgcc tttttggtga agaattgtcg ctgaccagtt cggttcagca ggtgatcggc 1560
ctggcaggta aggcaacgga catgtcggag tcttacagcc gagaggcaaa taaggacctg 1620
gtgttcatgg atatgaaaaa acttgcccaa ttcctcgcag gcaagcctga gcatccgatg 1680
accagagaaa cgcttaacgc cgaaaatatc gccaagtatg cttttagaat agtcccctga 1740
```

[0045] The nucleic acid sequence corresponding to SEQ ID NO:5 encodes a bacterial effector protein identified herein as AvrPtoB (H Pst PT23), which has a deduced amino acid sequence corresponding to SEQ ID NO:6 as follows:

```
Met Ala Gly Ile Asn Gly Ala Gly Pro Ser Gly Ala Tyr Phe Val Gly
  1               5                  10                  15

His Thr Asp Pro Glu Pro Ala Ser Gly Gly Ala His Gly Ser Ser Ser
             20                  25                  30

Gly Ala Ser Ser Ser Asn Ser Pro Arg Leu Pro Ala Pro Pro Asp Ala
         35                  40                  45

Pro Ala Ser Gln Ala Arg Asp Arg Arg Glu Met Leu Leu Arg Ala Arg
     50                  55                  60

Pro Leu Ser Arg Gln Thr Arg Glu Trp Val Ala Gln Gly Met Pro Pro
 65                  70                  75                  80

Thr Ala Glu Ala Gly Val Pro Ile Arg Pro Gln Glu Ser Ala Glu Ala
                85                  90                  95

Ala Ala Pro Gln Ala Arg Ala Glu Glu Arg His Thr Pro Glu Ala Asp
            100                 105                 110

Ala Ala Ala Ser His Val Arg Thr Glu Gly Gly Arg Thr Pro Gln Ala
        115                 120                 125

Leu Ala Gly Thr Ser Pro Arg His Thr Gly Ala Val Pro His Ala Asn
    130                 135                 140

Arg Ile Val Gln Gln Leu Val Asp Ala Gly Ala Asp Leu Ala Gly Ile
145                 150                 155                 160

Asn Thr Met Ile Asp Asn Ala Met Arg Arg His Ala Ile Ala Leu Pro
                165                 170                 175

Ser Arg Thr Val Gln Ser Ile Leu Ile Glu His Phe Pro His Leu Leu
            180                 185                 190

Ala Gly Glu Leu Ile Ser Gly Ser Glu Leu Ala Thr Ala Phe Arg Ala
        195                 200                 205

Ala Leu Arg Arg Glu Val Arg Gln Gln Glu Ala Ser Ala Pro Pro Arg
    210                 215                 220
```

```
Thr Thr Ala Arg Ser Ser Val Arg Thr Pro Glu Arg Ser Thr Val Pro
225                 230                 235                 240

Pro Thr Ser Thr Glu Ser Ser Ser Gly Ser Asn Gln Arg Thr Leu Leu
                245                 250                 255

Gly Arg Phe Ala Gly Leu Met Thr Pro Asn Gln Arg Arg Pro Ser Ser
            260                 265                 270

Ala Ser Asn Ala Ser Ala Ser Gln Arg Pro Val Asp Arg Ser Pro Pro
        275                 280                 285

Arg Val Asn Gln Val Pro Thr Gly Ala Asn Arg Val Val Met Arg Asn
    290                 295                 300

His Gly Asn Asn Glu Ala Asp Ala Ala Leu Gln Gly Leu Ala Gln Gln
305                 310                 315                 320

Gly Val Asp Met Glu Asp Leu Arg Ala Ala Leu Glu Arg His Ile Leu
                325                 330                 335

His Arg Arg Pro Ile Pro Met Asp Ile Ala Tyr Ala Leu Gln Gly Val
            340                 345                 350

Gly Ile Ala Pro Ser Ile Asp Thr Gly Glu Ser Leu Met Glu Xaa Pro
        355                 360                 365

Leu Met Asn Leu Ser Val Ala Leu His Arg Ala Leu Gly Pro Arg Pro
    370                 375                 380

Ala Arg Ala Gln Ala Pro Arg Pro Ala Val Pro Val Ala Pro Ala Thr
385                 390                 395                 400

Val Ser Arg Arg Pro Asp Ser Ala Arg Ala Thr Arg Leu Gln Val Ile
                405                 410                 415

Pro Ala Arg Glu Asp Tyr Glu Asn Asn Val Ala Tyr Gly Val Arg Leu
            420                 425                 430

Leu Ser Leu Asn Pro Gly Ala Trp Val Arg Glu Thr Val Ala Ala Phe
        435                 440                 445

Val Asn Asn Arg Tyr Glu Arg Gln Ala Val Val Ala Asp Ile Arg Ala
    450                 455                 460

Ala Leu Asn Leu Ser Lys Gln Phe Asn Lys Leu Arg Thr Val Ser Lys
465                 470                 475                 480

Ala Asp Ala Ala Ser Asn Lys Pro Gly Phe Lys Asp Leu Ala Asp His
                485                 490                 495

Pro Asp Asp Ala Thr Gln Cys Leu Phe Gly Glu Glu Leu Ser Leu Thr
            500                 505                 510

Ser Ser Val Gln Gln Val Ile Gly Leu Ala Gly Lys Ala Thr Asp Met
        515                 520                 525
```

```
Ser Glu Ser Tyr Ser Arg Glu Ala Asn Lys Asp Leu Val Phe Met Asp
    530                 535                 540

Met Lys Lys Leu Ala Gln Phe Leu Ala Gly Lys Pro Glu His Pro Met
545                 550                 555                 560

Thr Arg Glu Thr Leu Asn Ala Glu Asn Ile Ala Lys Tyr Ala Phe Arg
                565                 570                 575

Ile Val Pro
```

This bacterial effector protein has a molecular mass from 55-65 kDa.

[0046] Another suitable bacterial effector protein of the present invention is identified herein as *avrPtoB (H Pst JL1065)* and is encoded by a nucleic acid molecule having a nucleotide sequence of SEQ ID NO:7 as follows:

```
atggcgggta tcaatggagc gggaccatcg ggcgcttatt ttgttggcca cacagacccc 60
gagccagcat cgggggcgc acacggatcc agcagtggcg caagctcctc gaacagtccg 120
cgcttgccgg cgcctccgga tgcacccgcg tcgcaggcgc gagatcgacg cgaaatgctt 180
ttgcgagcca ggccgctgtc gcgccaaacc agggagtggg tggcgcaggg tatgccgcca 240
acggcggagg ctggagtgcc catcaggccg caggagtctg ccgaggctgc agcgccgcag 300
gcacgtgcag aggaaagaca cacgccggag gctgatgcag cagcgtcgca tgtacgcaca 360
gagggaggac gcacaccgca ggcgcttgcc ggtacctccc cacgccacac aggtgcggtg 420
ccacacgcca atagaattgt tcaacaattg gttgacgcgg gcgctgatct tgccggtatt 480
aacaccatga ttgacaatgc catgcgtcgc cacgcgatag ctcttccttc tcgaacagta 540
cagagtattt tgatcgagca tttccctcac ctgctagcgg gtgaactcat tagtggctca 600
gagctcgcta ccgcgttccg tgcggctctc cgtcgggagg ttcgccaaca ggaggcgtca 660
gccccccccaa gaacagcagc gcggtcctcc gtaaggacgc cggagcggtc gacggtgccg 720
cccacttcta cggaatcatc atcgggcagc aaccagcgta cgttattagg gcggttcgcc 780
gggttgatga cgcctaatca gagacgtccg tcgagcgctt cgaacgcgtc tgcctctcaa 840
aggcctgtag acagaagccc gccacgcgta aaccaggtac ccacaggcgc taacagggtt 900
gtgatgcgta atcatggtaa taacgaggcc gacgccgcgc tgcaaggatt ggctcagcag 960
ggggttgata tggaggacct gcgcgccgcg cttgaaagac atatattgca tcgccgcccg 1020
atccccatgg atatagcgta cgccttgcag ggtgtgggca ttgcgccaag tatcgatacg 1080
ggagagagcc ttatggaaaa cccgctgatg aatttgagtg ttgcgctgca ccgcgcacta 1140
gggcctcgtc ccgctcgtgc tcaagcgcct cgtccagccg ttccggtggc tcccgcgacc 1200
gtctccaggc gaccagatag cgcgcgtgcc acaagattgc aggtaatacc ggcgcgggag 1260
gattacgaaa ataatgtggc ctacggagtg cgcttgctga gcctgaatcc gggcgcgggg 1320
gtcagggaga ctgttgcggc ctttgtaaac aaccgttacg agcggcaggc ggttgttgcc 1380
gacatacgcg cagccctaaa tttatctaaa caattcaata agttgcgtac ggtctctaag 1440
gccgatgctg cctccaataa accgggcttc aaggatctgg cggaccaccc agacgacgcg 1500
acgcaatgcc tttttggtga agaattgtcg ctgaccagtt cggttcagca ggtgatcggc 1560
ctggcaggta aggcaacgga catgtcggag tcttacagcc gagaggcaaa taaggacctg 1620
gtgttcatgg atatgaaaaa acttgcccaa ttcctcgcag gcaagcctga gcatccgatg 1680
accagagaaa cgcttaacgc cgaaaatatc gccaagtatg cttttagaat agtcccctga 1740
```

[0047] The nucleic acid sequence corresponding to SEQ ID NO:7 encodes a bacterial effector protein identified herein as AvrPtoB (H Pst JL1065), which has a deduced amino acid sequence corresponding to SEQ ID NO:8 as follows:

```
Met Ala Gly Ile Asn Gly Ala Gly Pro Ser Gly Ala Tyr Phe Val Gly
  1               5                  10                  15

His Thr Asp Pro Glu Pro Ala Ser Gly Gly Ala His Gly Ser Ser Ser
```

20 25 30

Gly Ala Ser Ser Ser Asn Ser Pro Arg Leu Pro Ala Pro Pro Asp Ala
35 40 45

Pro Ala Ser Gln Ala Arg Asp Arg Arg Glu Met Leu Leu Arg Ala Arg
50 55 60

Pro Leu Ser Arg Gln Thr Arg Glu Trp Val Ala Gln Gly Met Pro Pro
65 70 75 80

Thr Ala Glu Ala Gly Val Pro Ile Arg Pro Gln Glu Ser Ala Glu Ala
85 90 95

Ala Ala Pro Gln Ala Arg Ala Glu Glu Arg His Thr Pro Glu Ala Asp
100 105 110

Ala Ala Ala Ser His Val Arg Thr Glu Gly Gly Arg Thr Pro Gln Ala
115 120 125

Leu Ala Gly Thr Ser Pro Arg His Thr Gly Ala Val Pro His Ala Asn
130 135 140

Arg Ile Val Gln Gln Leu Val Asp Ala Gly Ala Asp Leu Ala Gly Ile
145 150 155 160

Asn Thr Met Ile Asp Asn Ala Met Arg Arg His Ala Ile Ala Leu Pro
165 170 175

Ser Arg Thr Val Gln Ser Ile Leu Ile Glu His Phe Pro His Leu Leu
180 185 190

Ala Gly Glu Leu Ile Ser Gly Ser Glu Leu Ala Thr Ala Phe Arg Ala
195 200 205

Ala Leu Arg Arg Glu Val Arg Gln Gln Glu Ala Ser Ala Pro Pro Arg
210 215 220

Thr Ala Ala Arg Ser Ser Val Arg Thr Pro Glu Arg Ser Thr Val Pro
225 230 235 240

Pro Thr Ser Thr Glu Ser Ser Ser Gly Ser Asn Gln Arg Thr Leu Leu
245 250 255

Gly Arg Phe Ala Gly Leu Met Thr Pro Asn Gln Arg Arg Pro Ser Ser
260 265 270

Ala Ser Asn Ala Ser Ala Ser Gln Arg Pro Val Asp Arg Ser Pro Pro
275 280 285

Arg Val Asn Gln Val Pro Thr Gly Ala Asn Arg Val Val Met Arg Asn
290 295 300

His Gly Asn Asn Glu Ala Asp Ala Ala Leu Gln Gly Leu Ala Gln Gln
305 310 315 320

Gly Val Asp Met Glu Asp Leu Arg Ala Ala Leu Glu Arg His Ile Leu
325 330 335

His Arg Arg Pro Ile Pro Met Asp Ile Ala Tyr Ala Leu Gln Gly Val
340 345 350

Gly Ile Ala Pro Ser Ile Asp Thr Gly Glu Ser Leu Met Glu Asn Pro
355 360 365

Leu Met Asn Leu Ser Val Ala Leu His Arg Ala Leu Gly Pro Arg Pro
370 375 380

Ala Arg Ala Gln Ala Pro Arg Pro Ala Val Pro Val Ala Pro Ala Thr
385 390 395 400

Val Ser Arg Arg Pro Asp Ser Ala Arg Ala Thr Arg Leu Gln Val Ile
405 410 415

Pro Ala Arg Glu Asp Tyr Glu Asn Asn Val Ala Tyr Gly Val Arg Leu
420 425 430

Leu Ser Leu Asn Pro Gly Ala Gly Val Arg Glu Thr Val Ala Ala Phe
435 440 445

Val Asn Asn Arg Tyr Glu Arg Gln Ala Val Val Ala Asp Ile Arg Ala
450 455 460

Ala Leu Asn Leu Ser Lys Gln Phe Asn Lys Leu Arg Thr Val Ser Lys
465 470 475 480

Ala Asp Ala Ala Ser Asn Lys Pro Gly Phe Lys Asp Leu Ala Asp His
485 490 495

Pro Asp Asp Ala Thr Gln Cys Leu Phe Gly Glu Glu Leu Ser Leu Thr
500 505 510

Ser Ser Val Gln Gln Val Ile Gly Leu Ala Gly Lys Ala Thr Asp Met
515 520 525

Ser Glu Ser Tyr Ser Arg Glu Ala Asn Lys Asp Leu Val Phe Met Asp
530 535 540

Met Lys Lys Leu Ala Gln Phe Leu Ala Gly Lys Pro Glu His Pro Met
545 550 555 560

Thr Arg Glu Thr Leu Asn Ala Glu Asn Ile Ala Lys Tyr Ala Phe Arg
565 570 575

Ile Val Pro

This bacterial effector protein has a molecular mass from 55-65 kDa.

[0048] Also suitable in all aspects of the present invention are bacterial effector proteins which have an amino acid sequence spanning amino acids 308 and 553 of *avrPtoB (PstDC3000)* (SEQ ID NO:2).

[0049] Based on the avrPtoB (PstDC3000) amino acid comparisons described in Figures 7A-E, regions of highly conserved amino acid sequences were identified. Identification of these regions further enabled identification of specific motifs throughout the conserved region of avrPtoB (PstDC3000). As a result of this analysis, several blocks of 5 or more identical amino acids were found as shown in Table 1 below.

Table 1

Location in avrPtoB (PstDC3000)	Motif
7-22	AGPSGAYFVGHTDPEP (SEQ ID NO:9)
32-40	SGASSSNSP (SEQ ID NO:10)
71-78	LSRQTREW (SEQ ID NO:11)
132-137	IVQQLV (SEQ ID NO:12)
221-225	SSSGS (SEQ ID NO:13)
254-264	PVDRSPPRVNQ (SEQ ID NO:14)
361-372	APRPAVPVAPAT (SEQ ID NO:15)
374-378	SRRPD (SEQ ID NO:16)
381-385	RATRL (SEQ ID NO:17)
391-405	REDYENNVAYGVRLL (SEQ ID NO:18)
417-421	VAAFV (SEQ ID NO:19)
434-438	IRAAL (SEQ ID NO:20)
452-456	SKADA (SEQ ID NO:21)
490-497	QQVIGLAG (SEQ ID NO:22)
516-553	FMDMKKLAQFLAGKPEHPMTRETLNAENIAKYAFRIVP (SEQ ID NO:23)

The information presented in Table 1 can be combined to define the protein of the present invention as having amino acid sequence of SEQ ID NO: 24 (with X being any amino acid) as follows:

(6X) **AGPSGAYFVGHTDPEP** (9X) **SGASSSNSP** (30X) **LSRQTREW** (53X) **IVQQLV** (83X) **SSSGS** (28X) **PVDRSPPRVNQ** (96X) **APRPAVPVAPAT** (X) **SRRPD** (2X) **RATRL** (5X) **REDYENNVAYGVRLL** (11X) **VAAFV** (12X) **IRAAL** (13X) **SKADA** (33X) **QQVIGLAG** (18X) **FMDMKKLAQFLAGKPEHPMTRETLNAENIAKYAFRIVP**

[0050] Also suitable in all aspects of the present invention are bacterial effector proteins which have an amino acid sequence spanning a C-terminus of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, or SEQ ID NO:8.

[0051] Fragments of the above bacterial effector proteins are encompassed by the present invention.

[0052] Suitable fragments can be produced by several means. In one method, subclones of the genes encoding the bacterial effector protein of the present invention are produced by conventional molecular genetic manipulation by subcloning gene fragments. The subclones then are expressed *in vitro* or *in vivo* in bacterial cells to yield a smaller protein or peptide.

[0053] In another approach, based on knowledge of the primary structure of the protein, fragments of a bacterial effector protein encoding gene may be synthesized by using the PCR technique together with specific sets of primers chosen to represent particular portions of the protein. These then would be cloned into an appropriate vector for increased expression of a truncated peptide or protein.

[0054] Chemical synthesis can also be used to make suitable fragments. Such a synthesis is carried out using known amino acid sequences for a bacterial effector protein being produced. Alternatively, subjecting a full length bacterial effector protein to high temperatures and pressures will produce fragments. These fragments can then be separated by conventional procedures (e.g., chromatography, SDS-PAGE).

[0055] Mutations or variants of the above polypeptides or proteins are encompassed by the present invention. Variants may be made by, for example, the deletion or addition of amino acids that have minimal influence on the properties, secondary structure, and hydropathic nature of a polypeptide or protein. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification, or identification of the polypeptide.

[0056] Also suitable as an isolated nucleic acid molecule according to the present invention is a nucleic acid molecule having a nucleotide sequence that is

at least 85% similar, to the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7 of the present invention by basic BLAST using default parameters analysis.

[0057] Suitable nucleic acid molecules are those that hybridize to a nucleic acid molecule comprising a nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7 of the present invention under stringent conditions. For the purposes of defining the level of stringency, reference can conveniently be made to Sambrook et al., Molecular Cloning: a Laboratory Manual, 2nd Edition, Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, at 11.45 (1989). An example of low stringency conditions is 4-6X SSC/0.1-0.5% w/v SDS at 37°-45°C for 2-3 hours. Depending on the source and concentration of the nucleic acid involved in the hybridization, alternative conditions of stringency may be employed such as medium stringent conditions. Examples of medium stringent conditions include 1-4X SSC/0.25% w/v SDS at ≥ 45°C for 2-3 hours. An example of high stringency conditions includes 0.1-1X SSC/0.1% w/v SDS at 60°C for 1-3 hours. The skilled artisan is aware of various parameters which may be altered during hybridization and washing and which will either maintain or change the stringency conditions. Other examples of high stringency conditions include: 4-5X SSC/0.1% w/v SDS at 54° C for 1-3 hours and 4X SSC at 65°C, followed by a washing in 0.1X SSC at 65°C for about one hour. Alternatively, an exemplary stringent hybridization condition is in 50% formamide, 4X SSC, at 42°C. Still another example of stringent conditions include hybridization at 62°C in 6X SSC, .05X BLOTTO, and washing at 2X SSC, 0.1% SDS at 62°C.

[0058] The precise conditions for any particular hybridization are left to those skilled in the art, because there are variables involved in nucleic acid hybridizations beyond those of the specific nucleic acid molecules to be hybridized that affect the choice of hybridization conditions. These variables include: the substrate used for nucleic acid hybridization (e.g., charged vs. non-charged membrane); the detection method used (e.g., radioactive vs. chemiluminescent); and the source and concentration of the nucleic acid involved in the hybridization. All of these variables are routinely taken into account by those skilled in the art prior to undertaking a nucleic acid hybridization procedure.

[0059] A bacterial effector protein of the present invention is preferably produced in purified form (e.g., at least about 85% pure) by conventional techniques. For example, a bacterial effector protein of the present invention may be secreted into the growth medium of recombinant host cells. To isolate the bacterial effector protein, a protocol involving a host cell such as *Escherichia coli* may be used, in which protocol the *E. coli* host cell carrying a recombinant plasmid is propagated, homogenized, and the homogenate is centrifuged to remove bacterial debris. The supernatant is then subjected to sequential ammonium sulfate precipitation. The fraction containing the bacterial effector protein of the present invention is subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the proteins or polypeptides. If necessary, the protein fraction may be further purified by high performance liquid chromatography ("HPLC").

[0060] Another aspect of the present invention pertains to host cells, transgenic plants, and transgenic plant seeds containing a nucleic acid molecule encoding a bacterial effector protein which inhibits programmed cell death in eukaryotes.

[0061] The present invention relates to a nucleic acid construct that contains a nucleic acid molecule encoding for a bacterial effector protein. This involves incorporating one or more of the nucleic acid molecules of the present invention, or a suitable portion thereof, into host cells using conventional recombinant DNA technology. Generally, this involves inserting the nucleic acid molecule into an expression system to which the nucleic acid molecule is heterologous (i.e. not normally present). The expression system contains the necessary elements for the transcription and translation of the inserted protein-coding sequences.

[0062] The present invention also relates to an expression vector containing a nucleic acid molecule encoding a bacterial effector protein of the present invention. The nucleic acid molecules of the present invention may be inserted into any of the many available expression vectors and cell systems using reagents that are well known in the art. In preparing a nucleic acid vector for expression, the various nucleic acid sequences may normally be inserted or substituted into a bacterial plasmid. Any convenient plasmid may be employed,

which will be characterized by having a bacterial replication system, a marker which allows for selection in a bacterium, and generally one or more unique, conveniently located restriction sites. Numerous plasmids, referred to as transformation vectors, are available for transformation. The selection of a vector will depend on the preferred transformation technique and target cells for transfection.

[0063] Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC1084, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, CA, which is hereby incorporated by reference in its entirety), pQE, pIH821, pGEX, pET series (see F.W. Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," Gene Expression Technology vol. 185 (1990), which is hereby incorporated by reference in its entirety), pCB201, and any derivatives thereof. Any appropriate vectors now known or later described for genetic transformation are suitable for use with the present invention. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The nucleic acid sequences are cloned into the vector using standard cloning procedures in the art, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Press, NY (1989), and Ausubel, F. M. et al. (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y., which are hereby incorporated by reference in their entirety.

[0064] U.S. Patent No. 4,237,224, issued to Cohen and Boyer, which is hereby incorporated by reference in its entirety, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including prokaryotic organisms and eukaryotic cells grown in tissue culture.

[0065] A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible

with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria. The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

[0066] Thus, certain "control elements" or "regulatory sequences" are also incorporated into the plasmid-vector constructs of the present invention. These include non-transcribed regions of the vector and 5' and 3' untranslated regions, which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and/or translation elements, including constitutive, inducible, and repressible promoters, as well as minimal 5' promoter elements may be used. A constitutive promoter is a promoter that directs expression of a gene throughout the development and life of an organism. An inducible promoter is a promoter that is capable of directly or indirectly activating transcription of one or more nucleic acid sequences or genes in response to an inducer. In the absence of an inducer, the nucleic acid sequences or genes will not be transcribed or will only be minimally transcribed.

[0067] The DNA sequences of eukaryotic promoters differ from those of prokaryotic promoters. Furthermore, eukaryotic promoters and accompanying genetic signals may not be recognized in or may not function in a prokaryotic system, and, further, prokaryotic promoters are not recognized and do not function in eukaryotic cells.

[0068] Promotors vary in their "strength" (i.e. their ability to promote transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promotors in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promotors may be used. For instance, when cloning in *E. coli*, its bacteriophages, or plasmids, promotors such as the T7 phage promoter, *lac* promotor, *trp* promotor, *rec*A promotor, ribosomal RNA promotor, the P_R and

P_L promotors of coliphage lambda and others, including but not limited, to *lac*UV5, *omp*F, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid *trp-lac*UV5 (*tac*) promotor or other *E. coli* promotors produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene.

[0069] Other examples of some constitutive promoters that are widely used for inducing expression of transgenes include the nopoline synthase (NOS) gene promoter, from *Agrobacterium tumefaciens*, (U.S. Patent No. 5,034,322 issued to Rogers et al., which is hereby incorporated by reference in its entirety), the cauliflower mosaic virus (CaMV) 35S and 19S promoters (U.S. Patent No. 5,352,605 issued to Fraley et al., which is hereby incorporated by reference in its entirety), the enhanced CaMV35S promoter ("enh CaMV35S"), the figwort mosaic virus full-length transcript promoter ("FMV35S"), those derived from any of the several actin genes, which are known to be expressed in most cells types (U.S. Patent No. 6,002,068 issued to Privalle et al., which is hereby incorporated by reference in its entirety), and the ubiquitin promoter, which is a gene product known to accumulate in many cell types. Examples of constitutive promoters for use in mammalian cells include the RSV promoter derived from Rous sarcoma virus, the CMV promoter derived from cytomegalovirus, ß-actin and other actin promoters, and the EF1α promoter derived from the cellular elongation factor 1α gene.

[0070] Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promoter unless specifically induced. In certain operations, the addition of specific inducers is necessary for efficient transcription of the inserted nucleic acid. For example, the *lac* operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as *trp*, *pro*, etc., are under different controls.

[0071] Other examples of some inducible promoters, induced, for examples by a chemical agent, such as a metabolite, growth regulator, herbicide or phenolic compound, or a physiological stress/physical means, such as cold, heat, salt, toxins, or through the action of a pathogen or disease agent such as a virus or fungus, include a glucocorticoid-inducible promoter (Schena et al., Proc. Natl.

Acad. Sci. 88:10421-5 (1991), which is hereby incorporated by reference in its entirety), the heat shock promoter ("Hsp"), IPTG or tetracycline ("Tet on" system), the metallothionine promoter, which is activated by heavy metal ions, and hormone-responsive promoters, which are activated by treatment of certain hormones. A host cell containing an inducible promoter may be exposed to an inducer by externally applying the inducer to the cell. In addition, "tissue-specific" promoters can be used, which are promoters that function in a tissue specific manner to regulate the gene of interest within selected tissues of the host. Examples of such tissue specific promoters include seed, flower, or root specific promoters as are well known in the field (e.g., U.S. Patent No. 5,750,385 to Shewmaker et al., which is hereby incorporated by reference in its entirety). Promoters of the nucleic acid construct of the present invention may be either homologous (derived from the same species as the host cell) or heterologous (derived from a different species than the host cell).

[0072] Specific initiation signals are also required for efficient gene transcription and translation in prokaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The nucleic acid expression vector, which contains a promoter, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in *E. coli* requires an SD sequence about 7-9 bases 5' to the initiation codon ("ATG") to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the *cro* gene or the *N* gene of coliphage lambda, or from the *E. coli* tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

[0073] The constructs of the present invention also include an operable 3' regulatory region, selected from among those which are capable of providing correct transcription termination and polyadenylation of mRNA for expression in the host cell of choice, operably linked to a DNA molecule which encodes for a protein of choice. A number of 3' regulatory regions are known in the art.

Virtually any 3' regulatory region known to be operable in the host cell of choice would suffice for proper expression of the coding sequence of the nucleic acid of the present invention.

[0074] In one aspect of the present invention, the nucleic acid molecule of the present invention is incorporated into an appropriate vector in the sense direction, such that the open reading frame is properly oriented for the expression of the encoded protein under control of a promoter of choice. This involves the inclusion of the appropriate regulatory elements into the DNA-vector construct. These include non-translated regions of the vector, useful promoters, and 5' and 3' untranslated regions which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used.

[0075] A nucleic acid molecule of the present invention, promoter of choice, an appropriate 3' regulatory region, and, if desired, a reporter gene, can be incorporated into a vector-expression system which contains the nucleic acids of the present invention, or suitable fragments thereof, using standard cloning techniques as described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Press, NY (1989), and Ausubel et al. (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y., which are hereby incorporated by reference in their entirety. The transcriptional and translational elements are operably linked to the nucleic acid molecule of the present invention or a fragment thereof, meaning that the resulting vector expresses the bacterial effector protein when placed in a suitable host cell.

[0076] Once an isolated nucleic acid molecule encoding a bacterial effector protein has been cloned into an expression vector, it is ready to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The nucleic acid sequences are cloned into the host cell using standard cloning procedures known in the art, as described by Sambrook et al., Molecular Cloning:

A Laboratory Manual, Second Edition, Cold Springs Laboratory, Cold Springs Harbor, New York (1989), which is hereby incorporated by reference in its entirety. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, insect, plant, invertebrate, eukaryote and the like.

[0077] Thus, the present invention also relates to a host cell incorporating one or more of the isolated nucleic acid molecules of the present invention. In one embodiment, the isolated nucleic acid molecule is heterologous to the host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host system, and using the various host cells described above.

[0078] Methods of transformation may result in transient or stable expression of the DNA under control of the promoter. Preferably, the nucleic acid of the present invention is stably inserted into the genome of the host cell as a result of the transformation, although transient expression can serve an important purpose.

[0079] One approach to transforming host cells with a nucleic acid molecule of the present invention is particle bombardment (also known as biolistic transformation) of the host cell. This can be accomplished in one of several ways. The first involves propelling inert or biologically active particles at cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford et al., which are hereby incorporated by reference in their entirety. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the heterologous DNA. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried bacterial cells containing the vector and heterologous DNA) can also be propelled into plant cells. Other variations of particle bombardment, now known or hereafter developed, can also be used.

[0080] Transient expression in protoplasts allows quantitative studies of gene expression, because the population of cells is very high (on the order of 10^6). To deliver DNA inside protoplasts, several methodologies have been proposed,

but the most common are electroporation (Fromm et al., Proc. Natl. Acad. Sci. USA 82:5824-5828 (1985), which is hereby incorporated by reference in its entirety) and polyethylene glycol (PEG) mediated DNA uptake (Krens et al., Nature 296:72-74 (1982), which is hereby incorporated by reference in its entirety). During electroporation, the DNA is introduced into the cell by means of a reversible change in the permeability of the cell membrane due to exposure to an electric field. PEG transformation introduces the DNA by changing the elasticity of the membranes. Unlike electroporation, PEG transformation does not require any special equipment and transformation efficiencies can be equally high. Another appropriate method of introducing the nucleic acid molecule of the present invention into a host cell is fusion of protoplasts with other entities, either minicells, cells, lysosomes, or other fusible lipid-surfaced bodies that contain the chimeric gene (Fraley, et al., Proc. Natl. Acad. Sci. USA 76:3348-52 (1979), which is hereby incorporated by reference in its entirety).

[0081] Stable transformants are preferable for the methods of the present invention. An appropriate method of stably introducing the nucleic acid molecule into plant cells is to infect a plant cell with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* previously transformed with a DNA construct of the present invention. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots or roots, and develop further into plants.

[0082] Plant tissues suitable for transformation include without limitation, floral buds, leaf tissue, root tissue, meristems, zygotic and somatic embryos, megaspores, callus, protoplasts, tassels, pollen, embryos, anthers, and the like. The means of transformation chosen is that most suited to the tissue to be transformed.

[0083] Suitable plants include dicots and monocots. Monocots suitable for the present invention include Poaceae (e.g. rice, wheat, barley, rye, and sorghum) Gramineae (e.g., grass, corn, grains, bamboo, and sugar cane), Liliaceae (e.g., onion, garlic, asparagus, tulips, hyacinths, day lily, and aloes), Bromeliaceae (e.g. pineapple), and Musaceae (e.g. banana). Examples of dicots suitable for the present invention include Cruciferae (e.g., mustards, cabbage, cauliflower, broccoli, brussel sprouts, kale, kohlrabi, turnip, and radish), Rosaceae (e.g., raspberry, strawberry, blackberry, peach, apple, pear, quince, cherry, almond,

plum, apricot, and rose), Vitaceae (e.g. grape), Leguminosae (e.g., pea, bean, peanut, alfalfa, clover, vetch, redbud, broom, wisteria, lupine, black locust, and acacia), Fabaceae (e.g. soybean), Malvaceae (e.g., cotton, okra, and mallow), Umbelliferae (e.g., carrot, parsley, parsnips, and hemlock), Solanaceae (e.g., potato, tomato, pepper, eggplant, tobacco, henbane, atropa, physalis, datura, and *Petunia*), Convolvulaceae (e.g. sweet potato), Cucurbitaceae (e.g., melon, squash, pumpkin, cucumber, and zucchini), Asteraceae (e.g. chicory), Chenopodiaceae (e.g. spinach), Apiaceae (e.g. celery), Compositae (e.g., sunflower, endive, artichoke, lettuce, safflower, aster, marigold, dandelions, sage brush, *Dalia*, *Chrysanthemum*, and *Zinna*), Brassiceae (e.g. *Arabidopsis thaliana*), Geraniaceae (e.g. pelargonium and *saintpaulia*), and Euphorbiaceae (e.g. poinsettia).

[0084] After transformation, the transformed plant cells can be selected and regenerated. Preferably, transformed cells are first identified using a selection marker simultaneously introduced into the host cells along with the DNA construct of the present invention. Suitable selection markers include, without limitation, markers encoding for antibiotic resistance, such as the *nptII* gene which confers kanamycin resistance (Fraley, et al., Proc. Natl. Acad. Sci. USA 80:4803-4807 (1983), which is hereby incorporated by reference in its entirety), and the genes which confer resistance to gentamycin, G418, hygromycin, streptomycin, spectinomycin, tetracycline, chloramphenicol, and the like. Any known antibiotic-resistance marker can be used to transform and select transformed host cells in accordance with the present invention. Cells or tissues are grown on a selection medium containing the appropriate antibiotic, whereby generally only those transformants expressing the antibiotic resistance marker continue to grow. Other types of markers are also suitable for inclusion in the expression cassette of the present invention. For example, a gene encoding for herbicide tolerance, such as tolerance to sulfonylurea is useful, or the *dhfr* gene, which confers resistance to methotrexate (Bourouis et al., EMBO J. 2:1099-1104 (1983), which is hereby incorporated by reference in its entirety). Similarly, "reporter genes," which encode for enzymes providing for production of a compound identifiable are suitable. The most widely used reporter gene for gene fusion experiments has been *uidA*, a gene from *Escherichia coli* that encodes the β-glucuronidase protein, also known as GUS (Jefferson et al., EMBO J. 6:3901-3907 (1987), which is

hereby incorporated by reference in its entirety). Similarly, enzymes providing for production of a compound identifiable by luminescence, such as luciferase, are useful. The selection marker employed will depend on the target species; for certain target species, different antibiotics, herbicide, or biosynthesis selection markers are preferred.

[0085] Once a recombinant plant cell or tissue has been obtained, it is possible to regenerate a full-grown plant therefrom. It is known that practically all plants can be regenerated from cultured cells or tissues. Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing transformed explants is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, embryo formation can be induced in the callus tissue. These embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is usually reproducible and repeatable.

[0086] Plant regeneration from cultured protoplasts is described in Evans, et al., Handbook of Plant Cell Cultures, Vol. 1: (MacMillan Publishing Co., New York, 1983); and Vasil I.R. (ed.), Cell Culture and Somatic Cell Genetics of Plants, Acad. Press, Orlando, Vol. I, 1984, and Vol. III (1986), which are hereby incorporated by reference in their entirety.

[0087] After the nucleic acid construct is stably incorporated in transgenic plants, it can be transferred to other plants by sexual crossing or by preparing cultivars. With respect to sexual crossing, any of a number of standard breeding techniques can be used depending upon the species to be crossed. Cultivars can be propagated in accord with common agricultural procedures known to those in the field. Alternatively, transgenic seeds or propagules (e.g., cuttings) are recovered from the transgenic plants. The seeds can then be planted in the soil and cultivated using conventional procedures to produce transgenic plants.

[0088] Another aspect of the present invention pertains to expression vectors, transgenic plants, and transgenic plant seeds containing a nucleic acid construct having a nucleic acid molecule encoding a first bacterial effector protein of the present invention coupled to a nucleic acid molecule producing a second protein toxic to eukaryotes.

[0089] In this aspect of the present invention, the nucleic acid construct includes a nucleic acid molecule encoding a first protein which is a bacterial effector protein of the present invention coupled to a nucleic acid molecule producing a second protein toxic to eukaryotes. Suitable nucleic acid molecules useful in this aspect of the present invention for the first protein include all those encoding the bacterial effector proteins described above. Suitable second proteins include, but are not limited to, the mouse protein *Bax* and the mutant kinase Pto (Y207D). Suitable methods of preparation of expression vectors, transformation of desired hosts, selection, and regeneration of transformants can be carried out as described above.

[0090] The present invention is also directed to a method of suppressing programmed cell death in eukaryotes. This method involves transforming a eukaryote with a nucleic acid encoding a bacterial effector protein which inhibits programmed cell death in eukaryotes. The eukaryote is then grown under conditions effective to suppress programmed cell death in the eukaryote. Suitable nucleic acid molecules useful in this aspect of the present invention include all those encoding the bacterial effector proteins described above. Suitable methods of preparation of expression vectors, transformation of desired hosts, selection, and regeneration of transformants can be carried out as described above.

[0091] A further aspect of the present invention relates to a method of delaying senescence in plants. This method includes transforming a plant with a nucleic acid encoding a bacterial effector protein which inhibits programmed cell death in eukaryotes. The plant is then grown under conditions effective to delay senescence in the plant. Suitable methods of preparation of expression vectors, transformation of desired hosts, selection, and regeneration of transformants can be carried out as described above. Suitable plants in accordance with this method of the present invention are described above.

[0092] Yet another aspect of the present invention relates to a method of increasing protein expression in plants. This method involves transforming a plant with a nucleic acid encoding a first bacterial effector protein which inhibits programmed cell death in eukaryotes and a second protein which is toxic to plants. The plant is grown under conditions effective to increase expression of the second protein in the plant. Suitable nucleic acid molecules useful in this aspect of the present invention include all those encoding the bacterial effector proteins described above. Suitable methods of preparation of expression vectors, transformation of desired hosts, selection, and regeneration of transformants can be carried out as described above. Suitable plants in accordance with this method of the present invention are described above.

[0093] Another aspect of the present invention relates to a method of stabilizing a transgenic plant producing a protein toxic to plants. This method involves providing a transgenic plant transduced with a nucleic acid molecule encoding a first bacterial effector protein and a nucleic acid molecule producing a protein toxic to plants. The plant is grown under conditions effective to stabilize the plant. Suitable nucleic acid molecules useful in this aspect of the present invention include all those encoding the bacterial effector proteins described above. Suitable methods of preparation of expression vectors, transformation of desired hosts, selection, and regeneration of transformants can be carried out as described above. Suitable plants in accordance with this method of the present invention are described above.

[0094] Yet another aspect of the present invention relates to a method of treating a subject for a condition mediated to treat the condition mediated by programmed cell death. Conditions which can be treated in accordance with this method include Parkinson's disease, Alzheimer's disease, hepatitis, acute liver injury, and inflammation. This method involves administering to the subject a bacterial effector protein which inhibits programmed cell death, as described above.

EXAMPLES

Example 1 -- *Agrobacterium*-Mediated Transient Expression

[0095] *Agrobacterium*-mediated transient expression was performed as described in Sessa et al., "Thr38 and Ser198 are Pto autophosphorylation sites required for the AvrPto-Pto-mediated hypersensitive response." *EMBO J.* 19: 2257-2269 (2000), which is hereby incorporated by reference in its entirety. Unless indicated otherwise, *A. tumefaciens* strain GV2260 was used to syringe-infiltrate tomato and *N. benthamiana* leaves at a final density of 0.1 and 0.4 OD_{600nm}, respectively. All genes were expressed from the constitutive 35S CaMV promoter, except for the mouse Bax protein that was expressed from a dexamethasone inducible promoter (Aoyama et al., "A glucocorticoid-mediated transcriptional induction system in transgenic plants." *Plant Journal*, 11: 605-612 (1997), which is hereby incorporated by reference in its entirety). Avr9 and Cf9 constructs and strains are as described in Van der Hoorn et al., "Agroinfiltration is a versatile tool that facilitates comparative analyses of Avr9/Cf-9-induced and Avr4/Cf-4-induced necrosis." *MPMI*, 13: 439-446 (2000), which is hereby incorporated by reference in its entirety. Co-expression experiments were performed by mixing *A. tumefaciens* cultures at equal ratios. For controls and to test responses in the absence of individual genes, *A. tumefaciens* carrying the appropriate empty vector replaced the missing component in the mixtures.

Example 2 -- Plasmid and Strain Construction

[0096] All AvrPtoB truncations were generated by PCR using the following primer sets:

Δ4, 2-26 5'GTAATGCAGCGCCTCCCTATC3' (SEQ ID NO:25) and
R5 5'TCAGGGGACTATTCTAAAAGC3' (SEQ ID NO:26);
Δ6, F1 5'ATGGCGGGTATCAATAGAGCG3' (SEQ ID NO:27) and
R4 5'TCACACCCGCAATCGTGTTGCAC3' (SEQ ID NO:28);
Δ7, F1 and R3 5'TCATACATGTCTTTCAAGGGCCG3' (SEQ ID NO:29).

Truncations were cloned into pCR2.1 (Invitrogen, Carlsbad, CA) and sequenced. For yeast two-hybrid bait constructs, the truncations were excised from pCR2.1

using *Eco*RI and subcloned into the *Eco*RI site of the pEG202 vector. Yeast two-hybrid analysis was performed as described by Kim et al., "Two distinct pseudomonas effector proteins interact with the pto kinase and activate plant immunity." *Cell*, 109: 589-598 (2002), which is hereby incorporated by reference in its entirety. For transient expression, the cloned truncations were excised from pCR2.1 using *Xba*I and *Spe*I enzymes and cloned into the *Xba*I site of the pBTEX binary vector.

[0097] DC3000 chromosomal truncations of AvrPtoB were generated using the pKnockout vector and methods as described in Windgassen et al., "Rapid gene inactivation in Pseudomonas aeruginosa." *FEMS Microbiol Lett*, 193: 201-205 (2000), which is hereby incorporated by reference in its entirety. Using an AvrPtoB template, 400-500 bp PCR products were generated using the following primers sets:

Mut1: A2MUT1F 5' GTATCAATAGAGCGGGACCATC3' (SEQ ID NO:30) and A2MUT1R 5' CACTGACCACTTGCTGAACG3' (SEQ ID NO:31);
Mut2, A2MUT2F: 5'TGTCGCGCCAAACCAGGGCGTG3' (SEQ ID NO:32) and A2MUT2R: 5'CCATCACCAGGGCAAACC3' (SEQ ID NO:33);
Mut3, A2MUT3F: 5'GTATCGTTCAGCAATTGGTCAGTG3' (SEQ ID NO:34) and A2MUT3R: 5'ACG CGTATGGGTCTTTGGTTG3' (SEQ ID NO:35);
Mut 5, A2MUT5F: 5'ACGATTGCGGGTGATGC3' (SEQ ID NO:36) and A2MUT5R: 5'CCTCTTGGCTGTAAGGCTGC3' (SEQ ID NO:37).

Each PCR product was cloned into pCR2.1, subcloned into pKnockout-G and introduced into DC3000 by triparental mating. After primary selection, plasmid insertion into the chromosome was verified by: i) PCR using T7 and 2-30 (5'ATGGCGGGTATCAATAGAGCGG3') (SEQ ID NO:38) primers, and ii) Southern blot analysis using *Pst*I digested genomic DNA and the *avrPtoB* ORF as a probe.

Example 3 -- Immunoblotting

[0098] Detection of proteins expressed in the *Agrobacterium*-mediated transient assay was performed using standard immunoblotting procedures. Briefly, 48 hours after agroinfiltration, two 1 cm^2 leaf discs were ground in 400 µl of protein extraction buffer, composed of PBS amended with 1% Triton-x and

plant protease inhibitor cocktail (Sigma, St. Louis, MS). Protein extracts were denatured and equal amounts of protein were electrophoresed on 12% polyacrylamide gels and transferred to PVDF membrane (Millipore Immobilon P, Bedford, MA) by electroblotting according to the manufacturer's recommendation (Biorad, Hercules, CA). HA-tagged proteins were detected using rat anti-HA primary antibody (Boehringer-Mannheim, Indianapolis, IN), HRP-conjugated anti-Rat Ig secondary antibody (Amersham-Pharmacia, Piscataway, NJ) and a chemiluminescent visualization kit (ECL Plus, Amersham-Pharmacia).

Example 4 -- Yeast Cell Death Assays

[0099] The *S. cerevisiae* strain EGY48 (MATa, *ura3, his3, trp1,* $lexA_{op}$(x6)-LEU2) was obtained from Clontech (Palo Alto, CA) and the growth, transformation and expression of genes was performed essentially as described by Kampranis et al., "A novel plant glutathione S-transferase/peroxidase suppresses Bax lethality in yeast." *J. Biol Chem* 22: 29207-29216 (2000), which is hereby incorporated by reference in its entirety. The EGY48 cells were grown in YPD medium containing 1% yeast extract, 2% Difco peptone, and 2% glucose. AvrPtoB was cloned under the control of a galactose inducible plasmid in the high-copy yeast expression vector p423 (Mumberg et al., "Regulatable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use for heterologous expression." *Nucleic Acids Res,* 22: 5767-5768 (1994), which is hereby incorporated by reference in its entirety) and the plasmid was transformed into EGY48. Cells were grown in synthetic dropout (SD) medium with 2% glucose lacking histidine (SD/glu/-his) to select for the presence of the plasmid. EGY48 cells containing AvrPtoB were grown overnight in SD/glu/-his. The cells were pelleted, washed and resuspended in SD medium containing 2% galactose and 1% raffinose as carbon sources (SD/gal-raff/-his), to induce expression of the fusion protein from the GAL1 promoter. After 6 hr of induction, cells were diluted to 0.05 $O.D._{600}$ and treated in one of the following ways. For chemical treatments, H_2O_2 or menadione were added at selected final concentrations in the medium and cultures were incubated at 30°C with vigorous shaking for 6 hr. For heat stress, yeast cells were incubated at 37°C for 30 min with vigorous shaking,

then transferred to a water bath at 50°C for 30 min and then returned to 30°C with vigorous shaking for 6 hr. Following these treatments, viability was determined by plate counting. Treated and untreated cells were sampled and spread onto YPD medium with 2% agar, then incubated at 30°C for 48 hr. The number of colony forming units (Cfu) from treated cells (both EGY48 and EGY48 carrying AvrPtoB) were compared to the Cfu of untreated cells. All experiments were repeated in triplicate.

Example 5 – Tomato Infection and Measurement of Pathogen Growth in Leaves

[0100] Rio Grande (RG) tomato lines with the following genotypes were used in this study: RG-PtoR (*Pto/Pto, Prf/Prf*), RG-prf3 (*Pto/Pto, prf3/prf3*), RG-pto11 (*pto11/pto11, Prf/Prf*), and RG-ptoS (*pto/pto, Prf/Prf*). Bacterial growth measurements from tomato leaves were performed as described by Tang et al., "Overexpression of Pto activates defense responses and confers broad resistance." *Plant Cell*, 11: 15-30 (1999), which is hereby incorporated by reference in its entirety. Briefly, *P. s.* pv. *tomato* DC3000 strains were grown overnight in King's B (KB) medium with appropriate antibiotics. Cultures were washed twice with 10mM $MgCl_2$ and resuspended in 10mM $MgCl_2$. Washed cultures were prepared for inoculation by diluting cultures to 10^4 cells/mL in 10mM $MgCl_2$ and 0.04% Silwet L-77 (Osi, Danbury, CT). Six-week-old tomato plants were inoculated by vacuum infiltration and kept in a greenhouse during the course of infection. Bacterial growth was measured by grinding two 1 cm^2 leaf discs in 10mM $MgCl_2$, and tissue samples were serially diluted, and plated on solid KB medium with antibiotics.

Example 6 – AvrPtoB Broadly Suppresses PCD in *N. benthamiana* Leaves

[0101] The signaling components necessary for Pto-mediated PCD are conserved in the wild tobacco species *Nicotiana benthamiana*, because *Agrobacterium*-mediated transient co-expression of AvrPto and Pto in *N. benthamiana* leaves causes HR-related cell death, as shown in Figure 1A (Scofield et al., "Molecular basis of gene-for-gene specificity in bacterial speck disease of

tomato." *Science* 274: 2063-2065 (1996); Sessa et al., "Signal recognition and transduction mediated by the tomato Pto kinase: a paradigm of innate immunity in plants." *Microbes Infect*, 2: 1591-1597 (2000), which are hereby incorporated by reference in their entirety). AvrPtoB, however, does not trigger cell death when co-expressed with Pto in *N. benthamiana*, as shown in Figure 1A. This observation was unexpected because from yeast two-hybrid interactions and expression in tomato, it is known that AvrPtoB can interact with Pto and initiate PCD. It had been hypothesized that AvrPtoB, although likely binding to Pto in *N. benthamiana*, may also block downstream signaling events that lead to PCD.

[0102] To test if AvrPtoB could suppress AvrPto/Pto-mediated PCD, AvrPto, AvrPtoB and Pto were co-expressed in *N. benthamiana* leaves and found that AvrPto/Pto-dependent cell death was suppressed, as shown in Figure 1A and in Figure 1B. Cell death suppression was stable and observed as long as two weeks after inoculation. Expression of the three proteins in plant leaves was verified by using HA epitope-tagged constructs of AvrPto, AvrPtoB, and Pto; all three proteins were detected together and separately by immunoblot, as shown in Figure 1C. A possible explanation for the observed cell death suppression was that AvrPtoB out-competed AvrPto for interaction with the Pto kinase. To examine this possibility, AvrPtoB and Pto(Y207D) were co-expressed. Pto(Y207D) is a mutant kinase that, independent of effector recognition, initiates PCD (Rathjen et al., "Constitutively active Pto induces a Prf-dependent hypersensitive response in the absence of avrPto." *Embo J*, 18: 3232-3240 (1999), which is hereby incorporated by reference in its entirety). Expression of AvrPtoB suppressed Pto(Y207D)-initiated cell death, as shown in Figure 1A. This observation suggests AvrPtoB acts downstream of Pto recognition when suppressing cell death.

[0103] The activity of AvrPtoB was further investigated by examining Avr9/Cf9-initiated PCD. The Avr9 avirulence protein is produced by the fungus *Cladosporium fulvum* and elicits immunity in tomato plants expressing the Cf9 R protein (Van Kan et al., "Cloning and characterization of cDNA of avirulence gene avr9 of the fungal pathogen Cladosporium fulvum, causal agent of tomato leaf mold." *MPMI*, 4: 52-59 (1991); Jones et al., "Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging." *Science*, 266:

789-793 (1994), which are hereby incorporated by reference in their entirety). Avr9 and Cf9 also cause HR-related cell death when they are transiently co-expressed in *N. benthamiana* (Van der Hoorn et al., "Agroinfiltration is a versatile tool that facilitates comparative analyses of Avr9/Cf-9-induced and Avr4/Cf-4-induced necrosis." *MPMI*, 13: 439-446 (2000), which is hereby incorporated by reference in its entirety). Cf9-dependent and Pto-dependent PCD differ in several ways. First, Pto requires the *Prf* gene to signal PCD whereas Cf9-dependent PCD does not require *Prf*. Also, in the transient assay, Cf9-initiated cell death is substantially delayed compared to Pto-initiated cell death. Co-expression of AvrPtoB with Avr9 and Cf9 inhibited Avr9/Cf9-dependent cell death in *N. benthamiana*, as shown in Figure 1A. This finding suggests that AvrPtoB-mediated suppression of PCD acts on a target downstream of a point where these two *R* gene signaling pathways converge.

[0104] Given its surprisingly broad inhibitory activity, was examined to determine if AvrPtoB acts generally on the process of PCD in *N. benthamiana*. The mouse protein Bax is a member of the Bcl-2 family of pro-apoptotic proteins and initiates PCD by disrupting the mitochondrion and causing the release of cytochrome c and other pro-apoptotic factors (Jurgensmeier et al., "Bax directly induces release of cytochrome c from isolated mitochondria." *Proc Natl Acad Sci U S A*, 95: 4997-5002 (1998), which is hereby incorporated by reference in its entirety). Expression of the Bax protein in plants has been found to initiate a rapid cell death response that closely resembles the HR (Kawai et al., "Mammalian Bax-induced plant cell death can be down-regulated by overexpression of Arabidopsis Bax Inhibitor-1 (AtBI-1)." *Proc Natl Acad Sci U S A*, 98: 12295-12300 (2001); Lacomme et al., "Bax-induced cell death in tobacco is similar to the hypersensitive response." *Proc Natl Acad Sci U S A*, 96: 7956-7961 (1999), which are hereby incorporated by reference in their entirety). In both plants and yeast, Bax-induced cell death is dependent on a C-terminal mitochondrion-targeting domain (Lacomme et al., "Bax-induced cell death in tobacco is similar to the hypersensitive response." *Proc Natl Acad Sci U S A*, 96: 7956-7961 (1999), which is hereby incorporated by reference in its entirety), suggesting a common PCD-initiating mechanism across kingdoms. The mouse Bax protein was transiently expressed in *N. benthamiana* under control of a promoter that is

inducible by the glucocorticoid hormone dexamethasone (Dex) (Aoyama et al., "A glucocorticoid-mediated transcriptional induction system in transgenic plants." *Plant Journal*, 11: 605-612 (1997), which is hereby incorporated by reference in its entirety). The *Bax* gene by itself or constitutive *avrPtoB* and inducible *Bax* genes were co-transformed into *N. benthamiana* leaves and *Bax* expression was induced 48 hours after agroinfiltration by spraying leaves daily with 30 μM Dex. After five days of Dex induction, cell death was observed in leaves expressing Bax alone, while cell death was not observed in leaves expressing Bax and AvrPtoB, as shown in Figure 1A. The ability of AvrPtoB to broadly suppress PCD initiated by two distinct R proteins as well as the pro-apoptotic mouse protein Bax, suggests that AvrPtoB acts generally as an inhibitor of PCD in *N. benthamiana*.

Example 7 – AvrPtoB Suppresses PCD in Yeast

[0105] Since AvrPtoB broadly suppressed PCD in *N. benthamiana*, AvrPtoB was examined to determine if it may act on general components of eukaryotic cell death execution and perhaps AvrPtoB anti-PCD activity would be conserved in yeast. In *Saccharomyces cerevisiae*, PCD induced by oxidative stress or mammalian pro-apoptotic factors such as Bax, exhibits many of the hallmarks of metazoan apoptosis, including cytochrome c release, DNA fragmentation and chromatin condensation. As with mammalian apoptosis, oxidative stress is an important regulator of yeast PCD, and apoptotic responses can be induced by addition of low concentrations of hydrogen peroxide. AvrPtoB was expressed in the yeast strain EGY48 and yeast cells were treated with H_2O_2. Strikingly, it was observed that AvrPtoB protected yeast from PCD induced by 3 mM H_2O_2, as shown in Figure 2A and 2B, and 5 mM H_2O_2, as shown in Figure 2B. It was also found that AvrPtoB protected yeast from cell death induced by menadione and heat shock, as shown in Figure 2B. AvrPtoB, however, did not suppress Bax-induced cell death in yeast, suggesting that differences exist between Bax and AvrPtoB functions in *N. benthamiana* and yeast. The capacity of AvrPtoB to suppress PCD in plants and protect yeast from stress-induced PCD, clearly establishes AvrPtoB as a eukaryotic cell death inhibitor.

Example 8 – AvrPtoB has a Modular Structure with Distinct Recognition and Anti-PCD Domains

[0106] To better understand the basis of AvrPtoB recognition and anti-PCD functions a series of AvrPtoB N-and C-terminal truncations was constructed. Each of the truncations discussed in this study leads to an observable phenotype when expressed in plant leaves, thus establishing protein expression *in vivo*. AvrPtoB was examined to determine if it suppresses PCD but is still recognized by Pto, such that an AvrPtoB mutant could be developed such that the anti-PCD function was eliminated while the Pto recognition domain was maintained. In such a case, AvrPtoB/Pto-mediated cell death might be observed in *N. benthamiana*.

[0107] To map domains involved in AvrPtoB/Pto recognition, several deletion mutants were cloned as bait fusions and tested for interaction with a Pto prey fusion in a yeast two-hybrid system. Δ6 and Δ7 interacted strongly with Pto, as shown in Figure 3A. Therefore, an AvrPtoB fragment from amino acids 1-308 of SEQ ID NO:2 is sufficient for strong interaction with Pto in yeast.

[0108] The Pto-interacting AvrPtoB truncations were expressed in tomato and *N. benthamiana* to test for Pto-dependent cell death. As predicted from the yeast two-hybrid interaction, Δ7 triggered cell death in a *Pto*- and *Prf*-dependent manner in tomato, as shown in Figure 3B. In *N. benthamiana*, co-expression of Δ7 and Pto also resulted in cell death, as shown in Figure 4A. This gain of Δ7/Pto-initiated PCD demonstrates that the AvrPtoB N-terminus is sufficient for *in vivo* Pto-mediated recognition and suggests that the C-terminus is necessary for the observed PCD suppression. Significantly, intact AvrPtoB suppressed Δ7/Pto-initiated cell death when these three proteins were co-expressed, as shown in Figure 4B. Given that i) AvrPtoB was shown to act downstream of recognition for PCD suppression, and ii) full length AvrPtoB dominantly suppresses Δ7/Pto-initiated PCD, it is proposed that the N-terminal domain of AvrPtoB is recognized by the Pto kinase in *N. benthamiana*, but that the C-terminus of the same protein suppresses PCD signaled by this recognition event.

[0109] The newly observed Δ7/Pto-initiated PCD suggested that anti-PCD activity may reside in the AvrPtoB C-terminus. Several N-terminal deletions were tested for anti-PCD activity in *N. benthamiana*. Δ4 was found to be capable of

inhibiting cell death initiated by AvrPto/Pto, as shown in Figure 4A, Pto(Y207D) and Avr9/Cf9 in *N. benthamiana*. However, Δ4 PCD suppression was not as stable as full length AvrPtoB, often breaking down after seven days. Also, Δ4 did not suppress Bax-induced cell death, which is the most rapid and severe of the cell death phenotypes examined. The weaker anti-PCD function may be the result of altered localization, decreased protein stability or lower expression of the truncated form. Nevertheless, these data show that the C-terminus of AvrPtoB is sufficient for PCD inhibition. As such, recognition and anti-PCD functions could be separated into two non-overlapping AvrPtoB regions. Therefore, AvrPtoB has a modular structure with Pto-recognition in the N-terminal module and anti-PCD function in the C-terminal module.

Example 9 – Truncated AvrPtoB Elicits a Novel Resistance Phenotype, *Rsb*

[0110] When testing Δ6 for recognition activity in tomato and *N. benthamiana*, it was unexpectedly discovered that this truncation triggered PCD in the absence of Pto. In tomato plants that have a mutant *pto* gene, RG-pto11 (Salmeron et al., "Tomato mutants altered in bacterial disease resistance provide evidence for a new locus controlling pathogen recognition." *Plant Cell*, 6: 511-520 (1994), which is hereby incorporated by reference in its entirety), expression of Δ6 initiated rapid cell death, as shown in Figure 3B; however, in the absence of the *Prf* gene, Δ6 did not initiate cell death, as shown in Figure 3B, indicating that Δ6-mediated cell death is not the result of cytotoxicity. This new tomato resistance phenotype has been designated *Rsb* (Resistance Suppressed by AvrPtoB C-terminus). Similarly, Δ6 initiated cell death when expressed by itself in *N. benthamiana*, as shown in Figure 4A, demonstrating the conservation of the *Rsb* phenotype; *Rsb*-mediated cell death is also *Prf*-dependent in *N. benthamiana*. Interestingly, when Δ6 and Pto were co-expressed, a faster and more severe cell death phenotype was observed as compared to Δ6- or Δ7/Pto-initiated cell death, as shown in Figure 4A and in Figure 4B. This enhanced cell death phenotype may be indicative of multiple recognition events. Because Δ7 does not elicit Pto-independent cell death, a domain was mapped that triggers *Rsb*-mediated PCD between amino acids 308-388 of SEQ ID NO: 2.

[0111] Several explanations exist for the observed Δ6/*Rsb*-mediated PCD. One possibility is that the C-terminus of full length AvrPtoB physically hides the recognition domain, thus making it inaccessible to *Rsb*. Alternatively, full length AvrPtoB may normally suppress *Rsb*-initiated PCD downstream of *Rsb* recognition. AvrPtoB and Δ6 were co-expressed in *N. benthamiana* and tomato *pto* null mutants and in both cases PCD was not observed, as shown in Figure 4C, indicating that suppression of *Rsb*-dependent cell death occurs by an intermolecular mechanism. Moreover, intact AvrPtoB also suppressed the more severe Δ6/Pto-initiated PCD, as shown in Figure 4B. Given the evidence that AvrPtoB can act downstream of recognition for PCD suppression, it was proposed that intact AvrPtoB is recognized by a determinant of the *Rsb* resistance phenotype in tomato and *N. benthamiana*, but that the C-terminal module normally suppresses subsequent downstream events leading to PCD.

Example 10 – *AvrPtoB* is a Pathogenicity Factor that Induces Plant Susceptibility to P. s. pv. tomato DC3000 Infection

[0112] The discovery of *Rsb*-mediated PCD presented an opportunity to examine the role of PCD suppression in DC3000 pathogenesis. Since wild type DC3000 causes disease in RG-pto11 plants, it was hypothesized that intact AvrPtoB normally inhibits *Rsb*-mediated immunity in RG-pto11 tomato plants. Therefore, plant immunity might be elicited by a DC3000 mutant expressing an AvrPtoB C-terminal truncation where the anti-PCD function was destroyed but *Rsb* recognition was maintained. In parallel to this study, a series of C-terminal AvrPtoB truncations on the DC3000 chromosome was constructed by means of recombination with a plasmid by a single crossover event, as shown in Figure 5A. One of the mutants, DC3000::mut5, expressed an AvrPtoB fragment from amino acids 1-509, as shown in Figure 5A. Like wild type DC3000, DC3000::mut5 triggered immunity on RG-PtoR plants and caused disease on RG-prf3 plants, as shown in Figure 5B, in Figure 6A and in Figure 6B. However, like Δ6 in the transient assay, DC3000::mut5 triggered immunity when inoculated on RG-pto11 plants, as shown in Figure 5B, in Figure 6A and in Figure 6B. Wild type and mutant DC3000 strains with several other AvrPtoB chromosomal truncations did

not trigger immunity on RG-pto11, as shown in Figure 5B, demonstrating that the observed immunity is likely the result of the *Rsb* phenotype discovered in the transient assay.

[0113] To confirm that AvrPtoB acted as a pathogenicity determinant, DC3000::mut5 was transformed with the pDSK519 broad host range plasmid (Keen et al., "Improved Broad-Host-Range Plasmids for DNA Cloning in Gram-Negative Bacteria," *Gene* 70:191-197 (1988), which is hereby incorporated by reference in its entirety) expressing full length AvrPtoB from its native promoter. Expression of intact AvrPtoB *in trans* enabled DC3000::mut5 to cause disease in RG-pto11, as shown in Figure 6A and in Figure 6B. The observed DC3000::mut5-pDSK519::AvrPtoB disease symptoms were less severe than wild type DC3000, with approximately ten-fold less growth and smaller specks on the leaves. These slightly reduced disease symptoms are consistent with reported observations in *P. s.* pv. *maculicola* that effectors are sometimes better expressed from the chromosome than from a plasmid (Guttman et al., "Functional analysis of the type III effectors AvrRpt2 and AvrRpm1 of Pseudomonas syringae with the use of a single-copy genomic integration system." *Mol Plant Microbe Interact*, 14: 145-155 (2001), which is hereby incorporated by reference in its entirety). Nevertheless, addition of AvrPtoB was sufficient to shift the DC3000::mut5/RG-pto11 interaction from immunity to disease, demonstrating that AvrPtoB is a pathogenicity factor and that the final 44 amino acids of AvrPtoB are necessary to inhibit *Rsb*-mediated immunity. Interestingly, at four days after inoculation, DC3000::mut5 grew approximately ten-fold less on diseased RG-prf3 plants and caused less severe disease symptoms, when compared to wild type or DC3000::mut5 expressing intact AvrPtoB *in trans*. This observation hints that intact AvrPtoB may also act quantitatively as a virulence factor, perhaps by suppressing cell death. Because immunity was triggered by DC3000::mut5 and disease was regained with AvrPtoB expression *in trans*, and taken together with the findings that AvrPtoB acts downstream of recognition to inhibit PCD, these data suggest that AvrPtoB induces plant susceptibility to bacterial infection by inhibiting host PCD. Therefore, it was proposed that effector-mediated inhibition of PCD is an important novel bacterial pathogenesis strategy. Moreover, these data suggest that PCD is a necessary component of HR-based immunity in plants.

[0114] The discovery of *Rsb*-mediated immunity was an unexpected but useful tool to explore the role of AvrPtoB in plant disease. Although the *Rsb*-phenotype remains mostly uncharacterized, several clues point towards the basis of this immune response. First, the response was shown to be *Prf*-dependent, indicating it is likely a classical gene-for-gene resistance response. Given the observed Δ6-initiated HR in RG-PtoR and RG-pto11, and the absence of Δ6-initiated HR in RG-prf3 tomato plants, it was possible that *Prf* was the *Rsb* determinant. To examine this possibility, Δ6 was expressed in RG-ptoS tomato plants and also inoculated DC3000::mut5 on RG-ptoS plants. RG-ptoS is a near isogenic line with RG-PtoR and differs mainly at the introgressed Pto region, where RG-ptoS and RG-PtoR have the *L. esculentum* and *L. pimpinellifolium* Pto haplotypes, respectively (Martin et al., "Map-based cloning of a protein kinase gene conferring disease resistance in tomato." *Science*, 262: 1432-1436 (1993), which is hereby incorporated by reference in its entirety). RG-ptoS has a functional *Prf* gene, since ectopic expression of Pto in RG-ptoS plants leads to AvrPto-dependent cell death. Transient expression of Δ6 in RG-ptoS did not lead to HR, as shown in Figure 3B and DC3000::mut5 caused disease in RG-ptoS, as shown in Figure 5B. Together, these finding exclude *Prf* as the sole determinant of the *Rsb* phenotype and strongly indicate that the *Rsb* phenotype is governed by another gene (or genes) residing in the *L. pimpinellifolium* Pto region.

Example 11 – *Pseudonomas* type III Effector AvrPtoB Induces Plant Disease Susceptibility by Inhibition of Host Programmed Cell Death

[0115] It has been shown that the *P. s.* pv. *tomato* DC3000 type III effector AvrPtoB is a pathogenicity factor that can suppress HR-based plant immunity. By means of transient expression of individual proteins, inhibition of plant PCD was identified as the pathogenic mechanism of action of AvrPtoB. Given the presumed importance of PCD in HR-based plant defense, it is logical that a type III effector would target this process to induce host susceptibility. It is possible that other type III effectors that have been implicated in allowing plant pathogens to evade HR-based resistance (*e.g.* VirPphA, AvrPphC, and AvrPphF) also function using a similar mechanism. Previous to this work, several hypotheses had been proposed for the molecular basis of effector-mediated

evasion of the HR. The data present a conceptual stride forward in understanding the role of type III effectors in facilitating bacterial pathogenicity, and offer several new and interesting insights into the molecular basis of plant susceptibility and immunity.

[0116] AvrPtoB suppresses PCD in *N. benthamiana* triggered by two distinct R proteins and the pro-apoptotic mouse protein Bax and also suppresses cell death in yeast triggered by hydrogen peroxide, menadione and heat shock. Given its broad anti-PCD activity, AvrPtoB likely acts on a target far downstream in the process of HR and PCD signaling. AvrPtoB may act to suppress PCD by directly interfering with a host component necessary for PCD or by altering host gene expression or cell physiology to stimulate a PCD suppressive cellular environment. The molecular basis of plant PCD is still poorly characterized and few components that are known to control metazoan PCD have been characterized for plant PCD. Suppressors of plant PCD, however, have been identified, including pharmacological agents such as caspase inhibitors (del Pozo et al., "Caspases and programmed cell death in the hypersensitive response of plants to pathogens." *Curr Biol*, 8: 1129-1132 (1998); Lam et al., "Caspase-like protease involvement in the control of plant cell death." *Plant Mol Biol*, 44: 417-428 (2000), which are hereby incorporated by reference in their entirety) and in *Arabidopsis*, the At-BI1 protein, that was identified as a general suppressor of Bax triggered PCD in both yeast and *Arabidopsis* (Kawai et al., "Evolutionally conserved plant homologue of the Bax inhibitor-1 (BI-1) gene capable of suppressing Bax-induced cell death in yeast." *FEBS Lett*, 464: 143-147 (1999); Kawai et al., "Mammalian Bax-induced plant cell death can be down-regulated by overexpression of Arabidopsis Bax Inhibitor-1 (AtBI-1)." *Proc Natl Acad Sci U S A*, 98: 12295-12300 (2001), which are hereby incorporated by reference in their entirety). These observations indicate that, although still uncharacterized, targets for PCD inhibition exist in plants. It will be interesting to use AvrPtoB as a tool to investigate PCD in plants and yeast and possibly in other eukaryotic systems, such as insect and mammalian cells. Yeast can be a powerful tool to study the virulence activity of bacterial effector proteins of mammalian pathogens. Given that little is known about plant PCD, a yeast model should accelerate further study

of the genetics, cell biology and biochemistry of AvrPtoB cell death inhibition in both yeast and plants.

[0117] Plant immunity is a multifaceted phenomenon associated with an array of physiological responses including defense gene induction, phytoalexin production, reactive oxygen species formation and HR-related PCD. Although PCD is widely believed to play a role in limiting pathogen growth, the importance of PCD in plant immunity is the subject of debate, and gene-for-gene based immunity without HR-like PCD has been proposed (Clough et al., "The Arabidopsis dnd1 "defense, no death" gene encodes a mutated cyclic nucleotide-gated ion channel." *Proc Natl Acad Sci USA*, 97: 9323-9328 (2000); Yu et al., "Gene-for-gene disease resistance without the hypersensitive response in *Arabidopsis dnd1* mutant." *Proc Natl Acad Sci USA*, 95: 7819-7824 (1998), which are hereby incorporated by reference in their entirety). The finding that AvrPtoB functions to suppress both HR-based immunity and PCD strongly suggests that PCD is an essential and perhaps key component of HR-based immunity to *P. s.* pv. *tomato* DC3000. Further study, however, of how AvrPtoB affects plant physiology and gene expression will be necessary to explore this hypothesis.

[0118] Suppression of PCD by a bacterial type III effector is a novel pathogenesis strategy. Modulation of host PCD, however, is clearly important for bacterial pathogenesis as it has been observed in numerous model systems. For example, induction of PCD by type III effectors has been associated with disease formation of animal pathogens, including *Yersinia* (Juris et al., "Yersinia effectors target mammalian signalling pathways." *Cell Microbiol*, 4: 201-211 (2002), which is hereby incorporated by reference in its entirety) and *Salmonella* (Knodler et al., "Salmonella and apoptosis: to live or let die?" *Microbes Infect*, 3: 1321-1326 (2001), which is hereby incorporated by reference in its entirety). Although not experimentally associated with type III effectors, inhibition of PCD has been described for animal pathogens including *Chlamydia* (Geng et al., "Chlamydia pneumoniae inhibits apoptosis in human peripheral blood mononuclear cells through induction of IL-10." *J Immunol*, 164: 5522-5529 (2000), which is hereby incorporated by reference in its entirety), *Neisseria* (Massari et al., "Neisseria meningitidis porin PorB interacts with mitochondria and protects cells from apoptosis." *Proc Natl Acad Sci U S A*, 97: 9070-9075 (2000), which is hereby

incorporated by reference in its entirety) and *Rickettsia* (Clifton et al., "NF-kappa B-dependent inhibition of apoptosis is essential for host cellsurvival during Rickettsia rickettsii infection." *Proc Natl Acad Sci USA*, 95: 4646-4651 (1998), which is hereby incorporated by reference in its entirety). Interestingly, *Chlamydia* has a TTSS and it is therefore possible that effector-mediated PCD suppression is a common bacterial pathogenesis strategy in both plant and animal disease.

[0119] Plant pathogen effectors were initially isolated as avirulence proteins based on their ability to elicit the HR and plant immunity. Given the strong selective pressure for a pathogen to lose a factor that triggers immunity, it is widely assumed that type III effectors must also play an important role in disease formation. This assumption is supported by the observation that the TTSS is required for disease formation and experimental evidence that effector proteins can improve pathogen growth on plants (Chang et al., "avrPto enhances growth and necrosis caused by Pseudomonas syringae pv.tomato in tomato lines lacking either Pto or Prf." *Mol Plant Microbe Interact*, 13: 568-571 (2000); Chen et al., "The Pseudomonas syringae avrRpt2 gene product promotes pathogen virulence from inside plant cells." *Mol Plant Microbe Interact*, 13: 1312-1321 (2000); Shan et al., "A cluster of mutations disrupt the avirulence but not the virulence function of AvrPto." *MPMI*, 13: 592-598 (2000), which are hereby incorporated by reference in their entirety). One of the longstanding questions of plant pathogen effector research has been if avirulence and virulence functions of an effector could be physically separated. Distinct N- and C-terminal domains of AvrPtoB have been identified that are sufficient for recognition and anti-PCD activity, respectively. The modular nature of AvrPtoB raises several important questions about AvrPtoB evolution and function. For example, given its modular nature, it is possible that AvrPtoB evolved from a fusion of two ancestral proteins. Supporting this observation, truncated homologs of AvrPtoB that only contain the N-terminal module have been identified in Nature, including *P. s.* pv. *maculicola* effectors HopPmaL and HopPmaN (Guttman et al., "A functional screen for the type III (Hrp) secretome of the plant pathogen Pseudomonas syringae." *Science*, 295: 1722-1726 (2002), which is hereby incorporated by reference in its entirety), and the *P. s.* pv. *tomato* JL1065 AvrPtoB homolog, as shown in Figures 7A-E.

Intriguingly, the conservation of the recognized N-terminal domain by itself or with the anti-PCD domain, suggests that this domain may also serve a function in virulence, otherwise it would not be maintained in the pathogen. In fact, preliminary evidence using the DC3000:mut mutants described in this paper, suggests that the recognized N-terminal domain of AvrPtoB does play a role in *P. s.* pv. *tomato* DC3000 virulence.

[0120] It is noteworthy that AvrPtoB inhibits Pto-initiated PCD in *N. benthamiana* but not in tomato. This observation reveals that tomato has evolved a novel resistance response that acts to suppress AvrPtoB anti-PCD activity. Tomato, however, is not completely recalcitrant to AvrPtoB PCD inhibition, because *Rsb*-mediated PCD and immunity can be suppressed in RG-pto11. Because RG-PtoR and RG-pto11 plants are isogenic, except at *pto*, this implicates the Pto R protein as a candidate factor that acts to suppress anti-PCD activity, perhaps by binding and sequestering AvrPtoB. However, Pto alone is not sufficient, since AvrPtoB can suppress Pto-dependent PCD in *N. benthamiana*. Therefore, in tomato, it is predicted that other factors act in conjunction with Pto to inhibit AvrPtoB anti-PCD function, as shown in Figure 8. Overall, the model suggests that a chimeric effector can function at multiple points in a plant immune response and can either elicit or suppress plant immunity depending on the host genetic background. Such host-specific mechanisms are likely widespread, given observations from the *P. s.* pv. *phaseolicola*-bean pathosystem, where the effector AvrPphF inhibits HR-based resistance in bean cv. Tendergreen but triggers immunity in bean cv. Canadian Wonder (Tsiamis et al., "Cultivar-specific avirulence and virulence functions assigned to avrPphF in Pseudomonas syringae pv. phaseolicola, the cause of bean halo-blight disease." *Embo J*, 19: 3204-3214 (2000), which is hereby incorporated by reference in its entirety). Isolating factors that suppress the anti-PCD activity of AvrPtoB may reveal new signaling components of plant disease resistance and offer novel strategies for crop protection.

[0121] It was reported previously that the AvrPtoB GINP motif, from amino acids 325-328, was involved in AvrPtoB/Pto-mediated recognition (Kim et al., "Two distinct pseudomonas effector proteins interact with the pto kinase and activate plant immunity." *Cell*, 109: 589-598 (2002), which is hereby incorporated

by reference in its entirety). This result was based on the observations that: i) point mutations in the GINP motif weakened the interaction of AvrPtoB with Pto in a yeast two-hybrid system; ii) *P .s.* pv. *tomato* PT11 expressing AvrPtoB with a mutation in the GINP motif did not elicit an HR or immunity on Pto expressing tomato plants; and iii) the GINP motif is conserved in the AvrPto effector and is required for AvrPto/Pto interaction (Shan et al., "A Cluster of Mutations Disrupt the Avirulence But Not the Virulence Function of AvrPto," *MPMI* 13:592-598 (2000), which is hereby incorporated by reference in its entirety). In this study, however, it was found that Δ7, an AvrPtoB truncation that does not contain the GINP motif, still interacted strongly with Pto and triggered Pto-dependent PCD in plants. These seemingly contradictory data may offer insight into structural aspects of AvrPtoB. Since an AvrPtoB truncation missing the GINP motif is sufficient for Pto recognition, but intact AvrPtoB requires the GINP motif for Pto recognition, it is suspected that the GINP motif plays a key role in maintaining the structure of full length AvrPtoB. Interestingly, when mutations are introduced into the AvrPto GINP motif, the virulence function of AvrPto is maintained, indicating that GINP mutations do not necessarily destabilize the global structure of an effector. Rather, the GINP motif may act to "present" a contact surface to the Pto kinase. Data reported in this paper indicate that the AvrPtoB/Pto contact surface resides between amino acids 1-308 of SEQ ID NO: 2.

[0122] The unusually broad conservation of the AvrPtoB type III effector in many plant pathogens suggests AvrPtoB-mediated suppression of PCD and immunity plays an important role in bacterial pathogenesis. Certainly, AvrPtoB will be a useful tool to dissect the molecular basis of plant R protein PCD signaling, which presently is poorly understood. AvrPtoB anti-PCD activity may also have biotechnological applications; for example, AvrPtoB may allow efficient transgenic expression of proteins that otherwise elicit host PCD or may function to alter PCD-dependent plant developmental processes, such as senescence. Further study of AvrPtoB structure and function should lead to new insights into the basis of effector-mediated PCD inhibition and host mechanisms that guard against PCD inhibition.

Example 12 -- Bacterial Strains

[0123] The *E. coli* strains DH5α and DH10B (Gibco-BRL, Grand Island, NY), *Agrobacterium tumefaciens* strains EH105 and GV2260, and *P. s. tomato* strains were used for plasmid maintenance, transgene delivery, or infection assays, respectively. Plasmids used were pBluescript SK(-) (Stratagene, La Jolla, CA), pCR2.1 (Invitrogen, Carlsbad, CA), and pDSK519 (Keen et al., "Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria." *Gene*, 70: 191-197 (1988), which is hereby incorporated by reference in its entirety). Isolates and transconjugants of *P. s. tomato* were grown on King's medium B (KB) agar at 30°C and *E. coli* strains on LB agar or in LB broth at 37°C.

Example 13 -- Yeast Two-Hybrid Library Development and Screening

[0124] Plasmids (pEG202, pJG4-5, pSH18-34, pRFHM-1, and pJK101) and yeast strain EGY48 (*ura3*, *his3*, *trp1*, LexAop-LEU2) were provided by R. Brent (Mass. General Hospital, Boston, MA), and basic procedures for the yeast two-hybrid system are described previously (Zhou et al., "The tomato gene Pti1 encodes a serine/threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response." *Cell* 83: 925-935 (1995), which is hereby incorporated by reference in its entirety). The *Pseudomonas* prey library was generated in a modified vector series based on pJG4-5. Three sets of sense/antisense oligonucleotides containing a unique *Cla*I site and based on the *Eco*RI and *Xho*I polylinker fragment of pBluescript SK(-) were created:

(1) 5'- GAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAG-3' (SEQ ID NO:39);
(2) 5'-GAATTC**gaattgg**GATATCAAGCTTATCGATACCGTCGACCTCGAG-3' (SEQ ID NO:40); and
(3) 5'-GAATTC**gaatt**GATATCAAGCTTATCGATACCGTCGACCTCGAG-3' (SEQ ID NO:41).

Oligonucleotides (2) and (3) contained the nucleotides shown in bold lower case to produce two additional reading frames. Complementing oligos were annealed and the fragments introduced into pJG4-5 using the *EcoR*I and *Xho*I restriction enzyme sites. The resulting plasmids, pJG4-5/Y0, pJG4-5/Y1, and pJG4-5/Y2, contain a unique *Cla*I site for cloning and each has a different reading frame.

[0125] Insert DNA for the prey library was prepared by partial digestion of *P. s. tomato* DC3000 genomic DNA with the enzymes *Aci*I, *Msp*I, *HinP1*I, or

*Taq*I. Ten μg of each digest was size fractionated on a 0.8% agarose gel and fragments of 500 to 3000 bp were recovered. The DNAs were used in twelve ligation reactions (three vectors x four enzyme digests). Each ligation was transformed into ultracompetent *E. coli* strain DH10B and yielded $>10^8$ transformants. An equal number of transformants derived from each of the twelve libraries were pooled, grown in LB for 3 hour at 37°C, harvested by centrifugation, and DNA was extracted. The pooled DNA was transformed into *Saccharomyces cerevisiae* strain EGY48, which contained a LexA-Pto bait construct and the *lacZ* reporter plasmid pSH18-34 (Zhou et al., "The tomato gene Pti1 encodes a serine/threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response." *Cell* 83: 925-935 (1995), which is hereby incorporated by reference in its entirety). Approximately $>10^7$ transformants grew on glucose medium lacking uracil, histidine, and tryptophan, and colonies were recovered in TE buffer containing 50% glycerol and stored at –80°C. Approximately 5 x 10^7 yeast cells were plated on 10-cm plates containing galactose agar medium lacking uracil, histidine, tryptophan, and leucine. 2,500 colonies, which appeared within 5 days, were collected, and assays on selective medium containing X-gal were performed. 180 candidates that were either strongly, moderately, or weakly blue on X-gal plates were chosen for plasmid rescue and further analysis.

Example 14 -- Constructs For Expression of AvrPtoB in *Pseudomonas* or Plant Cells

[0126] A cosmid library of DC3000 from Alan Collmer (Cornell Univ.) and screened using a AvrPtoB probe. A clone, pDC101, carrying a 37-kb insert was identified and a 6.0-kb *Pst*I fragment was found to have the entire AvrPtoB open reading frame and putative Hrp-box. A 2.1-kb fragment from this region was using primer pair (avrPto2-14: 5'-CGGAGGCGAACAGCCGAGCAG-3' (SEQ ID NO:42); avrPto2-3: 5'-GCAATTCGAAGTGGCAGTGA-3' (SEQ ID NO:43)) and cloned into pCR2.1 and then into the broad host range vector pDSK519, creating pDSK519::*avrPtoB*. All *avrPtoB* constructs were verified by sequencing. Triparental mating was used to mobilize pDSK519::*avrPtoB* DNA from *E. coli* DH5α into *P. s. tomato* strains. For expression in plant cells, primer

pair avrpto2-12 (5'-TTATGCTTTATTGGTATTTTTAGAGG-3') (SEQ ID NO:44) and avrpto2-3, or avrpto2-15 (5'-ATGGCGGGTATCAATAGAGC-3') (SEQ ID NO:45) and avrpto2-3 were used to amplify just the *avrPtoB* coding region. The sequences obtained were subcloned downstream of the CaMV 35S promoter in the vector pBTEX (Frederick et al., "Recognition specificity for the bacterial avirulence protein AvrPto is determined by Thr-204 in the activation loop of the tomato Pto kinase." *Molecular Cell.* 2: 241-245 (1998), which is hereby incorporated by reference in its entirety) and this construct was used for transient expression in plant leaves (see below). Site-directed mutagenesis of the *avrPtoB* sequence was performed in plasmid pJG4-5 or in pBTEX using the Quickchange kit from Stratagene (La Jolla, CA). The desired mutations were confirmed by sequencing.

Example 15 -- Determination of Disease Symptoms on Plant Leaves and Bacterial Populations in Liquid Culture

[0127] Tomato (*Lycopersicon esculentum*) plants of Rio Grande-PtoS (RG-PtoS; *pto/pto, Prf/Prf*), Rio Grande-PtoR (RG-PtoR; *Pto/Pto, Prf/Prf*), and the mutants RG-prf-3 (*Pto/Pto, prf/prf*), and RG-pto11 (*pto/pto, Prf/Prf*) were grown in a greenhouse (24°C, 14 hr day). Tomato leaves on 7- or 8-week-old plants were vacuum-infiltrated with *P. s. tomato* bacterial suspensions of 10^4 or 10^7 colony-forming units per milliliter (cfu/mL). In low-inoculum level experiments, symptoms of bacterial speck disease developed over a 3 to 6 day period after inoculation. In high-inoculum level experiments, the HR occurred within 30 hr. Bacterial growth in KB liquid medium was determined by monitoring optical density at 600 nm and by plating serial dilutions of bacteria.

Example 16 -- Agrobacterium-Mediated Transient Expression in Plant Leaves

[0128] AvrPtoB expression constructs in pBTEX were introduced by electroporation into *Agrobacterium tumefaciens* strain GV2260 for tomato. *Agrobacterium* for inoculation was grown in LB medium overnight and diluted into induction medium (50 mM MES pH5.6, 0.5% (w/v) glucose, 1.7 mM

NaH_2PO_4, 20 mM NH4Cl, 1.2 mM $MgSO_4$, 2 mM KCl, 17 μM $FeSO_4$, 70 μM $CaCl_2$ and 200 μM acetosyringone) to an OD_{600}=0.03. Bacterial suspensions were injected with a needle-less syringe into leaves of 7- to 8-week-old tomato plants. Inoculated tomato plants were kept in constant low light in the laboratory and *N. benthamiana* was maintained in the greenhouse.

Example 17 – Identification of *Pseudomonas* Proteins that Interact with the Pto Kinase

[0129] To identify potential effectors from *P. s. tomato* DC3000 that interact with the Pto kinase a yeast two-hybrid screen was performed by using the tomato Pto kinase as the bait and a pool of DC3000 prey libraries. Based on the DNA sequences, ten classes of bacterial genes were identified in this screen, as shown in Table 2 below.

Table 2: Pto-interacting Proteins from *Pseudomonas syringae* pv. tomato DC3000

Clone	Number retrieved	GenBank Match	Organism	E-value
PtiDC1	8	VirPphA (AF141883)	*Pseudomonas syringae* pv. *phaseolicola*	e-140
PtiDC2	13	Dihydrofolate reductase (gi:150520)	*Salmonella typhimurium*	3e-28
PtiDC3	6	No matching sequence		
PtiDC4	5	Inducible catalase (gi:1778585)	*Pseudomonas putida*	3e-66
PtiDC5	5	Protein-L-isoaspartate O-methyltransferase (gi:2120644)	*Pseudomonas aeruginosa*	3e-28
PtiDC6	4	Alginate lyase (AB018795)	*Halomonas marina*	3e-58
PtiDC7	4	nhaR transcriptional activator (prf: 1817175B)	*Salmonella enteritidis*	3e-13
PtiDC8	4	Ribosomal protein L11 methyltransferase (gi:1075231)	*Haemophilus influenzae*	3e-25
PtiDC9	3	Chromosome initiation inhibitor (gi: 1519235)	*Aeromonas salmonicida*	2e-10
PtiDC10	2	Putative transposase (gi:2996223)	*Yersinia pestis*	3e-10

Additional proteins that were retrieved from the yeast two-hybrid screen of the DC3000 genomic library using Pto as the bait.

For unknown reasons, AvrPto was not recovered. One Pto-interacting class, PtiDC1, which contained eight clones, shared sequence similarity with a

previously described virulence-related protein (see below) and is the focus of this paper. The eight PtiDC1 clones did not auto-activate the reporter genes and re-transformation of them into the yeast expressing the Pto bait allowed growth on Leu- medium and cleavage of X-gal, as shown in Figure 9A. Thus, the PtiDC1 clones encode a protein that interacts with Pto kinase in the yeast two-hybrid system.

Example 18 – PtiDC1 Sequence is Similar to *virPphA* from *P. s. phaseolicola*

[0130] The nucleotide sequences were determined for the eight PtiDC1 clones and revealed they carried inserts truncated at three distinct 5' ends but were otherwise identical, as shown in Figure 9B. Comparison of the nucleotide sequences of the PtiDC1 inserts to current databases showed similarity to the effector gene *virPphA* (GenBank No. AF141883) from *P. s. phaseolicola* (Jackson et al., "Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native plasmid in the bean pathogen Pseudomonas syringae pathovar phaseolicola." *Proc Natl Acad Sci USA*, 96: 10875-10880 (1999), which is hereby incorporated by reference in its entirety). The gene was designated *avrPtoB* because the initial phenotype associated with the PtiDC1 sequence was avirulence (see below), and because this designation denoted its functional similarity with *avrPto* (i.e. *avrPto*, originally isolated from *P. s. tomato* strain JL1065, is formally $avrPtoA_{JL1065}$).

[0131] A cosmid was recovered from a DC3000 library by using a PtiDC1 probe and a 6.0 kb *Pst*I fragment containing *avrPtoB* was subcloned and sequenced. The sequence revealed an open reading frame (ORF) spanning 1,659 bp, as shown in Figure 9B (GenBank Acc. No. AY074795) (SEQ ID NO:2) with 52% nucleotide identity to the *virPphA* gene. A putative Hrp box (GGAACT-N_{16}-CCAC) (SEQ ID NO:46) is located 85 nucleotides upstream of the predicted AUG initiation codon and conforms closely to a consensus Hrp box recently derived from a large set of effectors from DC3000 (Fouts, et al., "Genomewide identification of Pseudomonas syringae pv. tomato DC3000 promoters controlled by the HrpL alternative sigma factor." *Proc Natl Acad Sci USA*, 99: 2275-2280 (2002), which is hereby incorporated by reference in its entirety). In accordance

with this observation it was found that *avrPtoB* gene expression is induced in apoplast-mimicking medium and *in planta* in a *hrp*-dependent fashion.

[0132] The *avrPtoB* ORF produces a predicted protein of 553 amino acids with a molecular mass of 59 kDa. Putative amino acid sequence of AvrPtoB is 52% identical to VirPphA (BLASTP e value = e-140), as shown in Figure 9C. The truncation points in the PtiDC1 clones, as shown in Figure 9B, were found to remove the first 70, 112, or 121 amino acids of the AvrPtoB open reading frame. Database searches detected no sequence similarity between AvrPtoB and AvrPto. In addition, unlike AvrPto, the AvrPtoB protein has no myristylation motif immediately following the initiation methionine (Nimchuk et al., "Eukaryotic fatty acylation drives plasma membrane targeting and enhances function of several type III effector proteins from *Pseudomonas syringae*." *Cell.* 101: 353-363 (2000); Shan et al., "The *Pseudomonas* AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane." *Plant Cell* 12: 2323-2337 (2000), which are hereby incorporated by reference in their entirety). However, pattern searching with PIR (Protein Information Resource) detected a possible myristylation site near the N-terminus (i.e. MAGINRAG (SEQ ID NO: 47); consensus motif is G-{not EDRKHPFYW}-x(2)-[STAGCN]–{not P}) (SEQ ID NO: 48) and 10 myristylation motifs within the protein.

Example 19 – Interaction Specificity of AvrPtoB Protein for the Pto Kinase

[0133] Interaction specificity between AvrPto and Pto has been characterized extensively (Scofield et al., "Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato." *Science* 274: 2063-2065 (1996); Tang et al., "The avirulence protein AvrPto physically interacts with the Pto kinase." *Science* 274: 2060-2063 (1996), Frederick et al., "Recognition specificity for the bacterial avirulence protein AvrPto is determined by Thr-204 in the activation loop of the tomato Pto kinase." *Molecular Cell.* 2: 241-245 (1998), which are hereby incorporated by reference in their entirety). To initially compare the Pto-interaction specificity of AvrPtoB with AvrPto, the AvrPtoB prey PtiDC1Δ70 with several bait plasmids expressing kinases closely related to Pto were introduced into the yeast two-hybrid system, as shown in Figure 9A.

AvrPtoB did not interact with the Fen kinase (Martin et al., "A member of tomato *Pto* gene family confers sensitivity to fenthion resulting in rapid cell death." *Plant Cell* 6: 1543-1552 (1994), which is hereby incorporated by reference in its entirety), the Pti1 kinase (Zhou et al., "The tomato gene Pti1 encodes a serine/threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response." *Cell* 83: 925-935 (1995), which is hereby incorporated by reference in its entirety), or the LescPtoF kinase (Jia et al., "Alleles of Pto and Fen occur in bacterial speck-susceptible and fenthion-insensitive tomato cultivars and encode active protein kinase." *Plant Cell* 9: 61-73 (1997); Riely et al., "Ancient origin of pathogen recognition specificity conferred by the tomato disease resistance gene *Pto*." *Proc. Natl. Acad. Sci. USA* 98: 2059-2064 (2001), which are hereby incorporated by reference in their entirety).

[0134] Next, a series of chimeric Pto-Fen proteins and Pto mutants were examined that were used previously to show that Thr-204 in the Pto activation loop is required for AvrPto-Pto interaction (Tang et al., "The avirulence protein AvrPto physically interacts with the Pto kinase." *Science* 274: 2060-2063 (1996); Frederick et al., "Recognition specificity for the bacterial avirulence protein AvrPto is determined by Thr-204 in the activation loop of the tomato Pto kinase." *Molecular Cell.* 2: 241-245 (1998), which are hereby incorporated by reference in their entirety). AvrPtoB specifically interacted with chimera G and not with other chimeric proteins, as shown in Figure 10A (Tang et al., "The avirulence protein AvrPto physically interacts with the Pto kinase." *Science* 274: 2060-2063 (1996), which is hereby incorporated by reference in its entirety). Comparison of chimera G with the other chimeras implicated a region in Pto from amino acids 129 to 224 that is required for interaction with AvrPtoB. AvrPto also interacts with chimera G and elicits the HR in tomato plants expressing a chimeric G transgene (Tang et al., "The avirulence protein AvrPto physically interacts with the Pto kinase." *Science* 274: 2060-2063 (1996), which is hereby incorporated by reference in its entirety). Additional Pto-Fen chimeras that subdivide the Pto region spanning amino acids 113 to 217 were all found to interact with AvrPtoB as they do with AvrPto, as shown in Figure 10B (Frederick et al., "Recognition specificity for the bacterial avirulence protein AvrPto is determined by Thr-204 in the activation loop of the tomato Pto kinase." *Molecular Cell.* 2: 241-245 (1998), which is

hereby incorporated by reference in its entirety). AvrPtoB also interacted in an identical fashion as AvrPto with a large series of Pto and Fen mutants that previously served to define recognition specificity of Pto for AvrPto, as shown in Figure 10C, (Frederick et al., "Recognition specificity for the bacterial avirulence protein AvrPto is determined by Thr-204 in the activation loop of the tomato Pto kinase." *Molecular Cell.* 2: 241-245 (1998), which is hereby incorporated by reference in its entirety). Taken together, AvrPtoB interacts with identical specificity as AvrPto with the Pto variants and these interactions thus indicate that T204 also forms a key recognition determinant of Pto for the AvrPtoB protein.

[0135] Further indication of the interaction specificity of AvrPtoB for Pto was obtained by examining a series of Pto proteins which contain single amino acid substitutions for eight previously identified autophosphorylation sites (Sessa et al., "Signal recognition and transduction mediated by the tomato Pto kinase: a paradigm of innate immunity in plants." *Microbes Infect*, 2: 1591-1597 (2000), which is hereby incorporated by reference in its entirety) and four Pto paralogs from the wild tomato species *L. hirsutum* (Riely et al., "Ancient origin of pathogen recognition specificity conferred by the tomato disease resistance gene *Pto*." *Proc. Natl. Acad. Sci. USA* 98: 2059-2064 (2001), which is hereby incorporated by reference in its entirety). A mutation at Thr-38 of Pto, the main autophosphorylation site in this kinase, abolishes the interaction with AvrPtoB as it does with AvrPto; all other phosphorylation site mutants interact with both AvrPtoB and AvrPto. Among the Pto kinases from *L. hirsutum*, only LhirPtoE interacts with the AvrPtoB and AvrPto proteins. Together, these observations demonstrate remarkable, and biologically meaningful, interaction specificity of the AvrPtoB protein for the Pto kinase.

Example 20 – *AvrPtoB* Sequences are Conserved in at Least Three Genera of Bacterial Pathogens

[0136] To examine the distribution of *avrPtoB*-like sequences the gene was used to probe DNA blots containing genomic DNA from many *Pseudomonas* pathovars, and some *Xanthomonas* and *Erwinia* strains. It was discovered that sequences with homology to *avrPtoB* are present in certain strains of each of these three genera, as shown in Figure 11. Because some of these strains (i.e. T1 and

PT11) are virulent on *Pto*-expressing tomato leaves, as shown in Table 3 below, it has been concluded that not all of these *avrPtoB* sequences are recognized by Pto. Several of these DNA fragments were cloned and by partial sequence analysis have confirmed their relatedness to *avrPtoB*.

Table 3: Reaction of Tomato Leaves to Inoculation with *P. s.* pv. *tomato* Strains Expressing *avrPtoB*

P. s. pv. *tomato* strain	Disease reaction[a]	
	RG-PtoR	RG-PtoS
T1	+	+
T1(*avrPto*$_{JL1065}$)	-	+
T1(*avrPtoB*)	+	+
PT11	+	+
PT11(*avrPto*$_{JL1065}$)	-	+
PT11(*avrPtoB*)	-	+
PT11(*avrPtoB*^{I326T})	+	+
PT11(*avrPtoB*^{G333A})	-	+
Bakersfield	+	+
Bakersfield(*avrPto*$_{JL1065}$)	-	+
Bakersfield(*avrPtoB*)	-	+

[a] Leaves of 6-week old tomato plants RG-PtoR (*Pto/Pto*) or RG-PtoS (*pto/pto*) were vacuum infiltrated with 10^4 cfu/mL of the *Pseudomonas* strain indicated. Disease symptoms were recorded 5 days after inoculation. +, >40 specks per leaflet; - no specks observed.

Example 21 – Expression of AvrPtoB in several *P. s. tomato* Strains Elicits Resistance to Bacterial Speck Disease in Tomato

[0137] To determine if *P. s. tomato* strains carrying *avrPtoB* elicited *Pto*-specific disease resistance, three race 1 (virulent) strains of *P. s. tomato* (T1, PT11, and Bakersfield) that do not contain a functional *avrPto* gene were examined. The pDSK519::*avrPtoB* clone (or pDSK519::*avrPto* as a control) was introduced into these strains and a suspension of 10^4 cfu/mL was vacuum infiltrated into the leaves of resistant (RG-PtoR) or susceptible (RG-PtoS) tomato plants. As summarized in Table 3 above, no disease symptoms were observed on

RG-PtoR plants inoculated with strains PT11 or Bakersfield expressing *avrPtoB* while RG PtoS plants were susceptible to these strains whether or not they carried *avrPtoB*. Identical results were observed for the *avrPto*-expressing strains. Interestingly, strain T1 elicited resistance in RG-PtoR only when expressing *avrPto*. Overall, these results confirmed that when expressed in at least two virulent strains of the bacterial speck pathogen, *avrPtoB* triggers plant resistance responses in a *Pto*-specific manner.

Example 22 – AvrPtoB is Translocated by the Type III Secretion System to Plant Cells

[0138] The interaction of AvrPtoB with Pto and the Hrp-dependent expression of the gene suggested that AvrPtoB is an effector that travels the TTSS to gain access to the plant cell cytoplasm. To test if AvrPtoB is secreted by the TTSS a strain of *P. fluorescens* was used that carries the Hrp cluster from *P. s. syringae* strain 61. *P. fluorescens* was transformed with the pDSK519::*avrPtoB* plasmid. Infiltration of tomato leaves with this strain elicited a strong HR in the *Pto*-containing cultivar RG-PtoR but not in line RG-PtoS that lacks *Pto*, as shown in Figure 11A. Infiltrated leaves of two tomato lines that contain inactive alleles of *Pto* or *Prf* also did not show induction of the HR. A *P. fluorescens* strain carrying the Hrp cluster but lacking AvrPtoB did not elicit an HR in any of the tomato lines. These results indicate that AvrPtoB is translocated into plant cells via the type III secretion system and that it is recognized specifically by the *Pto* locus in a *Prf*-dependent manner.

Example 23 – Expression of *AvrPtoB* Inside Tomato Leaf Cells Elicits a *Pto*- and *Prf*-Dependent HR

[0139] Expression of many Avr proteins directly in plant cells elicits *R* gene specific defenses indicating that they are the sole bacterial determinants of an intracellular recognition mechanism. Whether *avrPtoB* activates *R* gene specific defense was tested from within the plant cell by infiltrating *A. tumefaciens* strain GV2260 containing a CaMV *35S*::*avrPtoB* construct into tomato leaves with or without a functional Pto pathway, as shown in Figure 12B. Tomato leaves of line

RG-PtoR exhibited an HR within 24 hours of infiltration whereas the other leaves did not. *A. tumefaciens* carrying the empty binary vector elicited no responses in any of the leaves.

[0140] To confirm that AvrPtoB is recognized in tomato leaves specifically by the Pto kinase (and not another member of the Pto family), two *A. tumefaciens* strains containing either a *35S::avrPtoB* construct or *35S::Pto* construct were prepared and infiltrated either separately or as a mixture into leaves of the susceptible *pto* mutant, RG-pto11. Tomato leaves infiltrated with *A. tumefaciens* carrying *35S::avrPtoB* alone exhibited no response in these leaves (this observation is in contrast to transient expression of *avrPto* which causes necrosis in susceptible tomato leaves; Chang et al., "avrPto enhances growth and necrosis caused by Pseudomonas syringae pv.tomato in tomato lines lacking either Pto or Prf." *Mol Plant Microbe Interact*, 13: 568-571 (2000), which is hereby incorporated by reference in its entirety). However, tomato leaves infiltrated with a mixture of the *35S::Pto* and *35S::avrPtoB* strains developed an HR within 24 hr, as shown in Figure 12C. Thus, AvrPtoB is specifically recognized in tomato leaves by the Pto kinase. An ancillary, but interesting, separate experiment revealed that infiltration of a mixture of *Agrobacterium* carrying *35S::avrPtoB* and *35S::Pto* into leaves of *Nicotiana benthamiana* or *N. tabacum* W38 did not elicit an HR. This is in contrast to similar experiments using AvrPto (Scofield et al., "Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato." *Science*, 274: 2063-2065 (1996); Frederick et al., "Recognition specificity for the bacterial avirulence protein AvrPto is determined by Thr-204 in the activation loop of the tomato Pto kinase." *Molecular Cell.* 2: 241-245 (1998), which are hereby incorporated by reference in their entirety) and might indicate that AvrPtoB requires a distinct host component(s) for Pto-mediated HR which is lacking in these *Nicotiana* species.

Example 24 – AvrPtoB and AvrPto Proteins are Similar in Several Dispersed Regions

[0141] Although searches of GenBank using BLASTN and BLASTX failed to reveal sequence similarity between AvrPtoB and AvrPto, an alignment of the two proteins using DNASTAR did reveal similarities in several dispersed

regions, as shown in Figure 13A. The similarities between the two proteins have been used to designate nine subregions, I – IX, as shown in Figure 13A.

[0142] Subregion I contains the putative myristylation site for AvrPto. This site is required for both avirulence and virulence activity of AvrPto but not for its physical interaction with Pto. As discussed above, AvrPtoB does not have a likely myristylation site (Nimchuk et al., "Eukaryotic fatty acylation drives plasma membrane targeting and enhances function of several type III effector proteins from *Pseudomonas syringae*." *Cell.* 101: 353-363 (2000), which is hereby incorporated by reference in its entirety). Subregion III of both AvrPto and AvrPtoB contains the consensus sequences "RxxLxxSxxLxRxxxE" (SEQ ID NO: 49) and "SxRxR (SEQ ID NO: 50)." Interestingly, the first sequence is also found in a similar location in the protein sequences of VirPphA from *P. s. phaseolicola* race 7, $AvrRpt2_{JL1065}$ from *P. s. tomato*, and in less conserved form in several other Avr proteins, as shown in Figure 13B. In AvrRpt2, this sequence lies in an N-terminus 7.5 kDa region which is essential for secretion and translocation, but not for *in planta* avirulence activity (Mudgett et al., "Characterization of the *Pseudomonas syringae* pv. *tomato* AvrRpt2 protein: demonstration of secretion and processing during bacterial pathogenesis." *Mol. Microbiol.* 32: 927-941 (1999), which is hereby incorporated by reference in its entirety). A substitution mutation (H54P) within this region, when introduced into AvrPto and expressed in *P. s. tomato* or *P. s. tabaci,* abolishes its HR-eliciting activity in *Pto*-expressing leaves (Chang et al., "Functional studies of the bacterial avirulence protein AvrPto by mutational analysis." *Mol. Plant-Microbe Interact.* 14: 451-459 (2001), which is hereby incorporated by reference in its entirety). However, AvrPto(H54P) interacts with Pto in the yeast two-hybrid system and, when expressed directly within the plant cell, elicits an HR in *N. benthamiana* expressing *CaMV35S::Pto* and (Chang et al., "Functional studies of the bacterial avirulence protein AvrPto by mutational analysis." *Mol. Plant-Microbe Interact.* 14: 451-459 (2001), which is hereby incorporated by reference in its entirety). Therefore, this subregion might play a role in secretion or in translocating AvrPto and AvrPtoB (and possibly other proteins that have this sequence) into the plant cell. Finally, it should be noted that the retrieval from the two-hybrid screen of

AvrPtoB proteins lacking the first 121 amino acids indicates that neither subregions I, II or III are necessary for Pto binding in yeast.

[0143] Subregion IV contains four shared residues and one of them, S94 of AvrPto, was previously found to be important for interaction of AvrPto with Pto and for recognition by Pto in tomato (but not tobacco) cells (Shan et al., "The *Pseudomonas* AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane." *Plant Cell* 12: 2323-2337 (2000), which is hereby incorporated by reference in its entirety). In AvrPto, this residue lies next to a sequence that constitutes subregion V in our alignment. Subregion V consists of four conserved residues, GINP. Shan et al., "A cluster of mutations disrupt the avirulence but not the virulence function of AvrPto." *MPMI,* 13: 592-598 (2000), which is hereby incorporated by reference in its entirety, reported that a substitution in AvrPto at I96 in this sequence, or at the nearby G99 abolished recognition by Pto in yeast and tomato cells (G99, however, is not conserved in AvrPtoB). VirPphA from *P. s. phaseolicola* also has the GINP sequence (Jackson et al., "Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native plasmid in the bean pathogen Pseudomonas syringae pathovar phaseolicola." *Proc Natl Acad Sci USA*, 96: 10875-10880 (1999), which is hereby incorporated by reference in its entirety) and it has been found this protein both interacts with Pto in our yeast two-hybrid system and elicits an HR when expressed transiently in Pto-containing tomato leaves. Based on these observations it was speculated that residues in subregion V might be required for interaction of AvrPtoB with Pto (see below).

[0144] Finally, the alignment of AvrPto and AvrPtoB revealed four other discrete regions of shared amino acids in the C-terminal region. Deletion of the C-terminal 40 amino acids of AvrPto does not affect its interaction with Pto in yeast (Chang et al., "Functional studies of the bacterial avirulence protein AvrPto by mutational analysis." *Mol. Plant-Microbe Interact.* 14: 451-459 (2001), which is hereby incorporated by reference in its entirety) and this suggests that subregions VIII and IX of AvrPtoB are not required for Pto interaction. Substitutions at N145, P146, S147, or S153 of AvrPto abolished its ability to elicit the HR in tobacco line W38 raising the possibility that another Pto-like R protein exists in that line (Shan et al., "The *Pseudomonas* AvrPto protein is differentially

recognized by tomato and tobacco and is localized to the plant plasma membrane." *Plant Cell* 12: 2323-2337 (2000), which is hereby incorporated by reference in its entirety). N511 and P512 of AvrPtoB might serve a similar function although AvrPtoB also has an NPSxxxxxS (SEQ ID NO: 51) motif near subregion V (i.e., N327, P328, S329, S335). It has been found that expression of AvrPtoB in W38 does not elicit the HR but whether this is due to the different locations of this motif in the proteins or some other reason is not known.

Example 25 – Subregion V of AvrPtoB Contains Recognition Determinants for Interaction with Pto

[0145] A series of point mutations in AvrPtoB was developed to determine if subregion V, which is required for AvrPto interaction with Pto (Shan et al., "The *Pseudomonas* AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane." *Plant Cell* 12: 2323-2337 (2000), which is hereby incorporated by reference in its entirety), is also required for the AvrPtoB-Pto interaction, as shown in Figure 14. Each AvrPtoB point mutant was co-expressed with Pto in the yeast two-hybrid system and activation of the *lacZ* reporter gene was measured. Expression of the mutant proteins was confirmed by western blots. Substitutions G325A, I326T, or N327A of AvrPtoB reduced the interaction with Pto as compared with wildtype AvrPtoB (SEQ ID NO:2), as shown in Figure 14. Point mutations in nearby residues D331 and G333, which do not correspond to AvrPto residues, resulted in *lacZ* expression that was not statistically different from wildtype AvrPtoB. Two of these mutated *avrPtoB* genes were transformed into the virulent *Pseudomonas* strain PTII and examined their avirulence activity on RG-PtoR and RG-PtoS tomato leaves. Consistent with the two-hybrid data, AvrPtoB(I326T) (SEQ ID NO:2) did not elicit disease resistance on *Pto*-expressing leaves while AvrPtoB(G333A) (SEQ ID NO:2) elicited *Pto*-specific defense, as shown in Table 3. Thus, subregion V of AvrPto and AvrPtoB plays an important role in the Pto interaction and HR-eliciting activity of these effectors.

Example 26 – Two Distinct *Pseudomonas* Effector Proteins Interact with the Pto Kinase and Activate Plant Immunity

[0146] A second *Pseudomonas* protein, AvrPtoB, was identified that interacts with the Pto kinase and elicits *Pto*-specific and *Prf*-dependent disease resistance in tomato leaves. Speculation that such a protein exists arose after it was found that deletion of AvrPto from *P. s. tomato* strains JL1065 or DC3000 did not alter the avirulence of these strains on *Pto*-expressing tomato leaves. It was hypothesized that, like AvrPto, this putative second effector might also interact directly with the Pto kinase in a yeast two-hybrid system. A cross-kingdom yeast two-hybrid screen was employed and it permitted rapid and efficient isolation of AvrPtoB. AvrPto and AvrPtoB proteins have exactly the same interaction specificity for Pto in the yeast two-hybrid system and despite many differences they share several small, discrete, subregions in common. Subregion V plays a key role in the interaction with the Pto kinase and it is possible that other subregions also have conserved roles. These findings demonstrate that distinct bacterial effector proteins interact with the Pto kinase by using a common structural mechanism.

[0147] A yeast two-hybrid screen involving 12 *Pseudomonas* genomic prey libraries and a Pto bait construct was used to isolate AvrPtoB. The *Pseudomonas* genome is about 6.6 Mb, and, therefore, the screening of 5×10^7 random prey clones with an average insert size of 1 kb provides a >99.9% probability of testing every *Pseudomonas* genome sequence in the proper reading frame at least once for interaction with Pto. By using the DC3000 genome sequence (www.tigr.org) each of the PtiDC clones recovered were examined, as shown in Table 2 above, and, so far, have observed a Hrp box upstream of only the AvrPtoB open reading frame. Thus, unless the type III pathway also secretes non-Hrp-regulated proteins, it is likely that the interactions with Pto of the other proteins that were identified are not biologically meaningful. Eight AvrPtoB clones were recovered including some that were missing up to 121 amino acids from the N terminus of AvrPtoB but no clones that were missing anything downstream of this point. Because of the high probability that many subfragments of AvrPtoB are present in the *Pseudomonas* prey libraries these

results suggest that structural features spanning the C-terminal 432 amino acids of AvrPtoB are required for its interaction with Pto.

[0148] Several lines of evidence indicate that AvrPtoB is an effector that plays a role in restricting host range of *Pseudomonas*. First, in common with all previously identified *Avr* genes the *avrPtoB* promoter contains a consensus Hrp box. As expected, expression of *avrPtoB* is induced by growth medium that simulates the apoplastic fluid of plant leaves and is controlled by the Hrp regulon. Secondly, it was shown that delivery of AvrPtoB from *P. fluorescens* to plant cells is strictly dependent upon the presence of the TTSS encoded by the Hrp cluster. Third, the delivery of AvrPtoB from two normally virulent *Pseudomonas* strains or by *Agrobacterium*-mediated expression in the plant cell is detectable based on the specific recognition of the protein by the Pto kinase. Because Pto is localized within the plant cell this observation indicates that, as with many other Avr proteins (reviewed in Kjemtrup et al., "Effector proteins of phytopathogenic bacteria: bifunctional signals in virulence and host recognition." *Curr. Opin. Microbiol.* 3: 73-78 (2000), which is hereby incorporated by reference in its entirety), AvrPtoB is active inside the plant cell. Finally, there is the similarity of AvrPtoB to the VirPphA protein. VirPphA was originally identified in a *P. s. phaseolicola* strain as a virulence factor, because it promotes watersoaking by the pathogen in a bean pod assay. It was subsequently found to confer avirulence to *P. s. phaseolicola* bacteria infiltrated into soybean leaves (Jackson et al., "Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native plasmid in the bean pathogen *Pseudomonas syringae* pathovar *phaseolicola*." *Proc Natl Acad Sci USA*. 96: 10875-10880 (1999), which is hereby incorporated by reference in its entirety). In a related study, it was found that AvrPtoB also promotes watersoaking in the bean pod assay and, therefore, has virulence activity, too. VirPphA also interacts with Pto in the yeast two-hybrid system and elicits a *Pto*-specific HR in tomato leaves. Thus, the alignment of the two proteins, as shown in Figure 9C should expedite the identification of key residues in each protein that play a role in avirulence and virulence.

[0149] It was found that *avrPtoB* did not confer avirulence on all *P. s. tomato* strains tested, as shown in Table 3 above. This is consistent with the fact

that AvrPtoB was not isolated previously by screening of DC3000 cosmids in a virulent strain of *Pseudomonas* (Ronald et al., "The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the Pto resistance gene." 174: 1604-1611 (1992), which is hereby incorporated by reference in its entirety). It is possible that another *Pseudomonas* protein (e.g. a chaperone) is required for the effective secretion or translocation of AvrPtoB from *Pseudomonas* and that this factor is not present in all *P. s.* tomato strains. It is also possible that expression of AvrPtoB in certain bacterial strains is "masked" as observed for some effectors in *P. s. phaseolicola* (Jackson et al., "Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native plasmid in the bean pathogen *Pseudomonas syringae* pathovar *phaseolicola*." *Proc Natl Acad Sci USA.* 96: 10875-10880 (1999), which is hereby incorporated by reference in its entirety).

[0150] AvrPto was previously found to interact with certain Pto variants, and these proteins were used to define residue T204 of Pto as a key determinant of recognition specificity for AvrPto (Frederick et al., "Recognition specificity for the bacterial avirulence protein AvrPto is determined by Thr-204 in the activation loop of the tomato Pto kinase." *Molecular Cell.* 2: 241-245 (1998), which is hereby incorporated by reference in its entirety). Remarkably, AvrPtoB interacts with the same Pto variants as AvrPto and, thus, T204 is also a key Pto determinant for interaction with AvrPtoB. AvrPtoB also interacts with the one AvrPto-interacting member of the Pto family isolated from a bacterial speck-resistant wild species of tomato, *Lycopersicon hirsutum.* These observations suggest that there has been selection in *Lycopersicon* spp. over a long period of time for Pto-kinases that specifically recognize a conserved feature present in both the AvrPto and AvrPtoB proteins.

[0151] Dual recognition specificity previously has been reported for three other plant R proteins (i.e. RPM1, RPP8/HRT, Mi1; for review see Dangl et al., "Plant pathogens and integrated defence responses to infection." *Nature* 411: 826-833 (2001), which is hereby incorporated by reference in its entirety). However, in none of these cases have the host and pathogen proteins been shown to interact directly. Thus, the dual (or perhaps even multiple) recognition specificity of R proteins may turn out to be a common feature of plant defense responses. This

notion is consistent with the recent report that *Arabidopsis* contains only 150 putative *R* loci (of the NB-LRR class) yet is likely defending itself against many thousands of potential plant pathogens (Dangl et al., "Plant pathogens and integrated defence responses to infection." *Nature* 411: 826-833 (2001), which is hereby incorporated by reference in its entirety). Although the pathogen proteins recognized by most of these R genes are unknown, the present work suggests that common structural motifs embedded within diverse pathogen proteins might play a role in their recognition. Finally, if the possibility that the Pto kinase originally might have been an important target for several bacterial virulence proteins is considered, then the data are also consistent with the "guard" hypothesis which postulates that NB-LRR proteins (e.g. Prf) have evolved to interact with a complex of Avr proteins and their virulence targets (Dangl et al., "Plant pathogens and integrated defence responses to infection." *Nature* 411: 826-833 (2001), which is hereby incorporated by reference in its entirety).

[0152] A detailed structure-function analysis of both Avr proteins will be necessary to fully understand the importance of residues conserved between them. This analysis began by examining subregion V (the "GINP motif"), because it is perfectly conserved in both AvrPto and AvrPtoB and previous work with AvrPto found that several residues within this subregion are required for interaction with Pto (Shan et al., "A cluster of mutations disrupt the avirulence but not the virulence function of AvrPto." *MPMI,* 13: 592-598 (2000), which is hereby incorporated by reference in its entirety). Substitutions in the three residues examined in subregion V significantly decreased interaction of AvrPtoB with Pto while substitutions just outside subregion V did not. These results, along with the previous findings with AvrPto, suggest that the GINP motif may play a role as contact point between the Pto kinase and these two effector proteins. Alternatively, the GINP motif could affect the structure of another part of these proteins that interacts with Pto. The three-dimensional structure of the AvrPto protein is currently being determined, and this will allow further examination of the role of the GINP motif in Pto recognition.

[0153] Although *avrPto*-like sequences occur only in a subset of *Pseudomonas* strains that are known to be avirulent on *Pto*-expressing tomato plants (Ronald et al., "The cloned avirulence gene *avrPto* induces disease

resistance in tomato cultivars containing the Pto resistance gene." 174: 1604-1611 (1992), which is hereby incorporated by reference in its entirety) *avrPtoB*-like sequences are present in at least three genera of bacterial phytopathogens, as shown in Figure 11. *AvrPtoB* is one of only a few known Avr genes to show this wide distribution (White et al., "Prospects for understanding avirulence gene function." *Curr.* Opin. Plant Biol. 3: 291-298 (2000), which is hereby incorporated by reference in its entirety). It might be anticipated that widely conserved effectors serve as virulence factors, and this appears to be the case for AvrPtoB. Several *avrPtoB*-related sequences have been cloned from selected *Pseudomonas*, *Erwinia,* and *Xanthomonas* strains and from preliminary sequence analysis find a high degree of similarity among them. Future study of the AvrPtoB/VirPphA family will reveal if it plays a conserved role in promoting virulence in these diverse phytopathogens.

[0154] Although these studies revealed many similarities between AvrPto and AvrPtoB, some striking and intriguing differences were also observed. First, are the differences in the genes and corresponding proteins. *AvrPtoB*-like sequences are widely distributed whereas *avrPto*-like sequences have not been observed outside of the *Pseudomonas* spp. The proteins encoded by each gene are very different with AvrPtoB, at 59 kD, over three times the mass of AvrPto at 18 kD. There are sequence similarities at both the N- and C termini of the proteins and the main additions of AvrPtoB lie within four large internal segments. It was also found that, unlike AvrPto, the AvrPtoB protein lacks a myristylation motif at the penultimate position of the N terminus. The myristylation motif of AvrPto is required for both its avirulence and virulence activity and also for association of AvrPto with the membrane fraction (Shan et al., "A cluster of mutations disrupt the avirulence but not the virulence function of AvrPto." *MPMI,* 13: 592-598 (2000), which is hereby incorporated by reference in its entirety). The possibility that AvrPtoB protein might be processed to reveal an internal myristylation motif like, the AvrPphB protein, cannot be excluded (Nimchuk et al., "Eukaryotic fatty acylation drives plasma membrane targeting and enhances function of several type III effector proteins from *Pseudomonas syringae*." *Cell.* 101: 353-363 (2000), which is hereby incorporated by reference in its entirety). However, in

preliminary experiments using an AvrPtoB::GFP fusion, the protein does not appear to localize specifically to the cell periphery.

[0155] The second major difference observed between AvrPto and AvrPtoB is their apparent activity in plant cells. Unlike *avrPto*, the expression of *avrPtoB* in susceptible tomato or *N. benthamiana* leaves does not cause severe yellowing and necrosis that is dependent on the presence of Prf (Chang et al., "avrPto enhances growth and necrosis caused by Pseudomonas syringae pv.tomato in tomato lines lacking either Pto or Prf." *Mol Plant Microbe Interact*, 13: 568-571 (2000), which is hereby incorporated by reference in its entirety). It is not clear whether this AvrPto-mediated necrosis is a defense or susceptibility response, but the lack of the response in leaves expressing AvrPtoB might indicate that the two proteins target different host proteins as susceptibility targets when Pto is not present. In this regard, it will be interesting to see if host proteins that are known to interact with AvrPto or the AvrPto-Pto complex will also do so with AvrPtoB or AvrPtoB-Pto (Bogdanove et al., "AvrPto-dependent Pto-interacting proteins and AvrPto-interacting proteins in tomato." *Proc. Natl. Acad. Sci. USA* 97: 8836-8840 (2000), which is hereby incorporated by reference in its entirety). Finally, it was surprising to discover that co-expression of AvrPtoB and Pto in leaves of *N. benthamiana* did not lead to an HR as does co-expression of AvrPto and Pto. This suggests that, although both effectors target the Pto kinase, they each may require additional and distinct host proteins for their avirulence activities.

[0156] Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.